Immune checkpoints as gatekeepers of $CD8^+$ T cell differentiation

by
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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland
January 2019

Abstract

Immune checkpoint inhibition represents a promising approach for multiple malignancies. Why successful T cell responses are achieved in only some patients is not well understood. We investigated how combined loss of PD-1 and LAG-3 signaling in $CD8^+$ T cells would influence a well-characterized immune response. After challenge with recombinant vaccinia virus expressing ovalbumin (VV-OVA), T cells from mice lacking both inhibitory pathways underwent a more vigorous primary effector cell response but were severely defective during a secondary challenge. Mechanistically, combined immune checkpoint inhibition resulted in greater production of irreversibly differentiated effector cell progeny, but at the expense of self-renewal of progenitor cells. Drugs that blunt anabolism-associated signaling corrected progenitor cell loss driven by combined checkpoint inhibition. Unleashing greater T cell activation may accelerate regenerative failure of critical clones. Fostering expansion of self-renewing progenitors using reversible agents that dampen T cell signal strength might paradoxically improve, not impede, the durability of immune checkpoint inhibition.

Acknowledgments

I would like to thank all the people of my lab who helped me with this work including: Dr. Charles Drake, Dr. Christina Ceccato Kochel, Dr. Christopher Nirschl, Dr. Daisy Shen, Debebe Theodros, Dr. Brian Francica, Dr. Yuki Muroyama, Dr. Ali Ghasemzadeh, Areli Lopez, Wendy Mao, Thomas Nirschl.

To my thesis committee and oral examination committee: Dr. Carolyn Machamer, Dr. Jonathan Powell, Dr. Fidel Zavala, Dr. Jay Bream, Dr. Giorgio Raimondi, Dr. Leo Luznik, Dr. Mark Soloski.

Others in my department at JHU: Dr. Joel Pomerantz, Dr. Abdel Hamad, Dr. Christopher Gamper, Dr. Fan Pan, Dr. Stuart Ray, Dr. Alan Scott, Shawn Rice, Angela James, Lori Fountain, Dr. Bob Leone, Dr. Brian Ladle, Dr. Jyoti Sen, Dr. Nicolas Llosa, Dr. Todd Armstrong, Dr. Rupashree Sen, Dr. Mario Amzel, Dr. Nicola Heller, Dr. Sarah McCormick, Dr. Min-Hee Oh, Im-Hong Sun, Dr. Franck Housseau, Dr. Andrea Cox, Dr. Diane E. Griffin, Dr. Nicola Heller, Dr. Matthew Arwood, Nelson Song, Dr. Zach Freeman, Dr. Scheherazade Sadegh-Nasseri, Dr. T. C. Wu., Dr. Sudipto Ganguly

Others at Johns Hopkins: Dr. Peter Espenshade, Dr. Douglas Robinson, Dr. Tyler Wied, Eileen Kim, Andrew Luksik, Raffaello Cimbro, Tomas Garzon-Mudvi, Trishia Nilles, Dr. Daniel Thorek, Dr. Jelani Zarif, Deepthi Ashok, Dr. Namandje Bumpus, Catherine Willl, Eric Lee.

My adoptive lab at Columbia University: Dr. Steve Reiner, Nyanza Rothman, Dr. Wen-Hsuan Wendy Lin, Dr. Radomir Kratchmarov, Dr. Bonnie Yen.

Others at Columbia U: Dr. Nato Teteloshvili, Dr. Remi Creusot, Mike Kisner. Dr. Debanjana Chatterjee, Dr. Rafael Gras Pena, Dr. Anna Cieslak, Michelle Miron, Dr. Rodney Macedo.

My other advisors and mentors: Dr. Lawrence B. Schramm, Dr. Carolyn Machamer, Dr. Daniel Teraguchi, Dr. Soveig Halldorsdottir, Dr. Giovanna Gerrero-Medina, Dr. Monica Feliu Mojer, Dr. Yaihara Fortis, Dr. Shashirekha Mundhra, Dr. Nilabh Shastri, Dr. Tamara O'Connor, Dr. Aaron Chandler, Dr. Meghan Pennini, Dr. Sofya Kerzhner, Dr. Adam Riess, Dr. Kimberly Bartsch, Dr. Andrew Kreuz, Professor Jimmy Manzano, Professor Lonnie McKenzey, Dr. Richard D. Bucher. Dr. Fred Paraskevoudakis, Dr. Fikire Wendweson, Dr. Ellen Roskes, Dr. Tracey Mason, Professor Sean Lally.

My family and friends: Vicente Haro, Ketty Haro, Enrique Carrera, Mgr. Diego Carrera-Haro, Mgr. Valeria Carrera-Haro, Lic. Marc Shapiro, Mgr. Marisol Angulo, Tulio Resende Baeta Zille, Dr. Darius Alix-Williams, Dr. Justin D. Glenn, Dr. Danya Ben-Hail, Danyel Addes, Nicholas Cornejo, Dr. David M. Loeb, Dr. Fiorela Protto, Penny Harris, Elliot Platt Esq., Roberta Platt Shapiro, Leonard Shapiro, Judy Nissenson Chant, Sonia Rustein, Darwin Quiroz, Dr. Rebecca Parodi-Rullan, Ana Sofia Racines, Daniel Paredes, Lic. Wendy Crespo, Lois Borgenicht, Joaquin E. Carrera, Rafaela Carrera, Amanda Buch, Fabiola Haro, Dr. Lizzy Skurnick, Dr. Maria Strangas, Dr. Lobsang Espinoza, Andrea Casildo, Alfredo Sota, Dr. Monica Rodriguez, Dr. Konomi Fujimory, Dr. Joao Rodrigo Souza Leao, Evelyn Marroquin Arch., Dr. Victor Serey, Amelia Beals, Dr. Julie Jurgens, Lic. Fredy Haro, Marco Garcia, Dr. Melania Bembea, Leonardo Silvestri, Marius and Pietro Silvestri, Hon. Nathan Braverman, Hon. Jamey Hueston, George Gardner, Dr. Melanie Brown, Henry Brown, Lydia Brown, Dr. Joe Brown, Maggie Brown, Roberto

Carlos Cerda Tapuy, Lic. Patricia Calderon, Sandra Espinoza, Dr. Valentina Cuccio, Lic. Xotchil Rodriguez, Lic. Marlene Felicita, Lic. Isabel Loyo, Dr. Nelson Segura, Lic. Juan Luis Siguenza, Luis Antonio Ron Serrano, Colin Busch, Will Busch, Kathleen Busch, Mauro Angel Moreno, Kemardo Henry, Mgr. Teresa Gras, Dr. Maria Royo.

To all the bars, museums and libraries specially Pocion Lounge in NYC, and Johnny Dee's Lounge in Baltimore.

… and finally I would like to thank all the people I forgot to thank above.

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List of abbreviations

PD-1: Programed cell death protein 1

LAG-3: Lymphocyte activation gene 3

VV-OVA: Recombinant vaccinia virus expressing ovalbumin

MPECs: Memory precursor effector cells

SLECs: Short lived effector cells

HSC: Hematopoietic stem cell

TCR: T cell receptor

APC: Antigen presenting cell

TCF1: Transcription factor T-cell factor 1

OXPHOS: Oxidative phosphorylation

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

PDL-1: Programmed death-ligand 1

ITSM: Immunoreceptor tyrosine-based switch motive

NK: Natural killer cells

SHP1: Src homology region 2 domain-containing phosphatase-1

SHP2: Protein-tyrosine phosphatase 1D

Chapter I: Introduction

Section 1.1: Immune curiosity, the vaccine that conquers 'Rose buds'*

The first notions of immunological memory

"Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience, and had now no fear for themselves; for the same man was never attacked twice" Thucydides (ca. 430 BCE).[1]

History tells us that ancient civilizations had recognized notions of disease. As early as 2000 BCE the Babylonian epic of Gilgamesh is not only the first written down literary work, but it narrates the existence of disease and pestilence.[2,3] Humans undoubtably are curious and since early times were observant and questioned about why disease could kill some while others survived. The curiosity for the concept of immunologic memory has been in the human mind through time and space. In the ancient Greece, the historian Thucydides registers that survivors of the plague did not get infected a second time.[4] The notion of "immunity" is first registered in the works of non-physician Greeks and in the same way the concept of immunity is used in the frame of disease first in Roma with those who had no training or practiced medicine.

* Reference to 'Upon the death of the Lord Hastings,' poem by John Dryden upon the death by smallpox of his schoolmate and friend Lord Hastings.[249]

The first registered use of the word immune in the human history, appears in Rome in the *Histories* by Polybious (200-117 BCE). In Latin *immunitas* and *immunis* originate from the legal concept of exception from a service or a duty.[5] It is until around 60 CE that *immunitas* is used to refer as exemption from disease, from lethal poison. Marcus Annaeus Lucanus in his poem *Pharsalia* describes the resistance to snake venom: " The nature of the country has arranged that, because living mixed with snakes, they are immune to them."[6]

The Chinese were the ones that not only observed but preformed the earliest attempt to immunize. They approach the treatment of smallpox through variolation.[7] The technique acquired popularity in the Ottoman Empire and from there it spread in Europe thanks to adventuress Lady Mary Worley Montagu .[8,9] Variolation helped to prevent smallpox. However, it was still unclear at that time how and why this "phenomena" was caused.

The unknown and limited control over variolation made of the technique a game of randomness. Of those variolated 1% to 2% died, which was still a gain considering that those who died after smallpox infection were 30%. [8,10] The idea behind variolation as a means to fight smallpox was used later in England by Jenner (1796 CE).[11] He was the first using vaccination and as a mean of prevention of disease and by far was safer than variolation.[12]

The idea of an acquired protection is long lived; however, the concept of immunological memory although pivotal for the study of the immune system, is relative recent. The scientific literature has recorded it as such during the 50's and 60's.[13,14] Immunological memory is one of the key concepts in the study of contemporary immunology.[15] How we define immunological memory continue to evolve and expand as we gain more understanding of the immune system.[15–17]

Section 1.2: $CD8^+$ T cells

$CD8^+$ T cell activation and effector function

$CD8^+$ T cells play a key role in the adaptive immune response to pathogens and cancer.[18,19] The precursors of T cells arise in the bone marrow and migrate to the thymus. Naïve $CD8^+$ T cells are generated in the thymus where they mature from hematopoietic stem cell (HSC) precursors.[20,21] After thymic selection, naïve $CD8^+$ T cells travel and seed in peripheral lymphoid tissue sites.[22] In the periphery and out of the thymus, naïve $CD8^+$ T cells are maintained through IL-7 and low-level tonic T cell receptor (TCR) signaling.[23]

Under pathogenic infection, naïve $CD8^+$ t cells can recognize foreign epitopes however, their initial frequency is low.[24] In the mouse for example, naïve $CD8^+$ T cells range between 10 and 3000 antigen-specific T cells per mouse.[25] $CD8^+$ T cells can expand to 10^4 to 10^5 in a period of 8 days.[24] All of this happens in specialized lymphoid organs where naïve $CD8^+$ T cells are recruited and seeded. It is crucial for $CD8^+$ T cells to rapidly expand, travel to the compromised sites and fight infection.[18,26,27]

There is a highly diverse repertoire of TCR in the naïve $CD8^+$ T cells pool. The TCR needs to engage with a cognate antigen in order to be activated. But TCR recognition is not enough. Once naïve $CD8^+$ T cells are stimulated by antigen presenting cells, they receive co-stimulation and interact with pro-inflammatory cytokines, they expand and

respond to infection (Fig. 1.1).[28–30] $CD8^+$ naïve T cells undergo considerable changes in shape and gene expression.[31,32]

A.

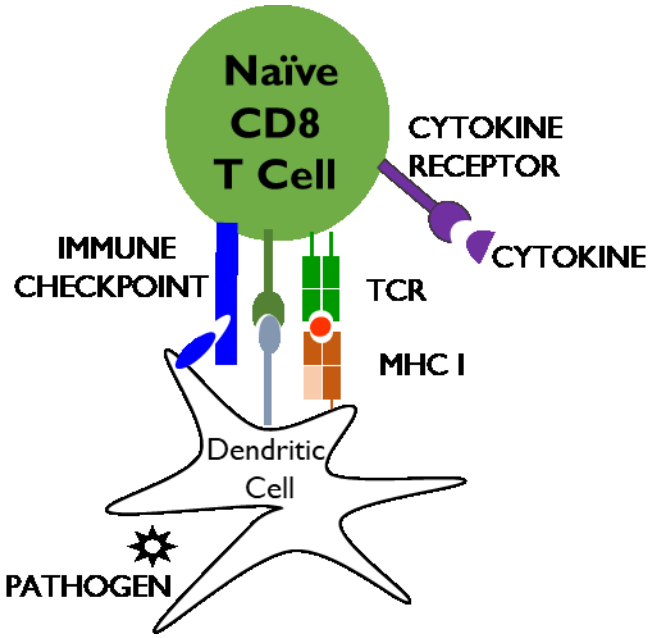
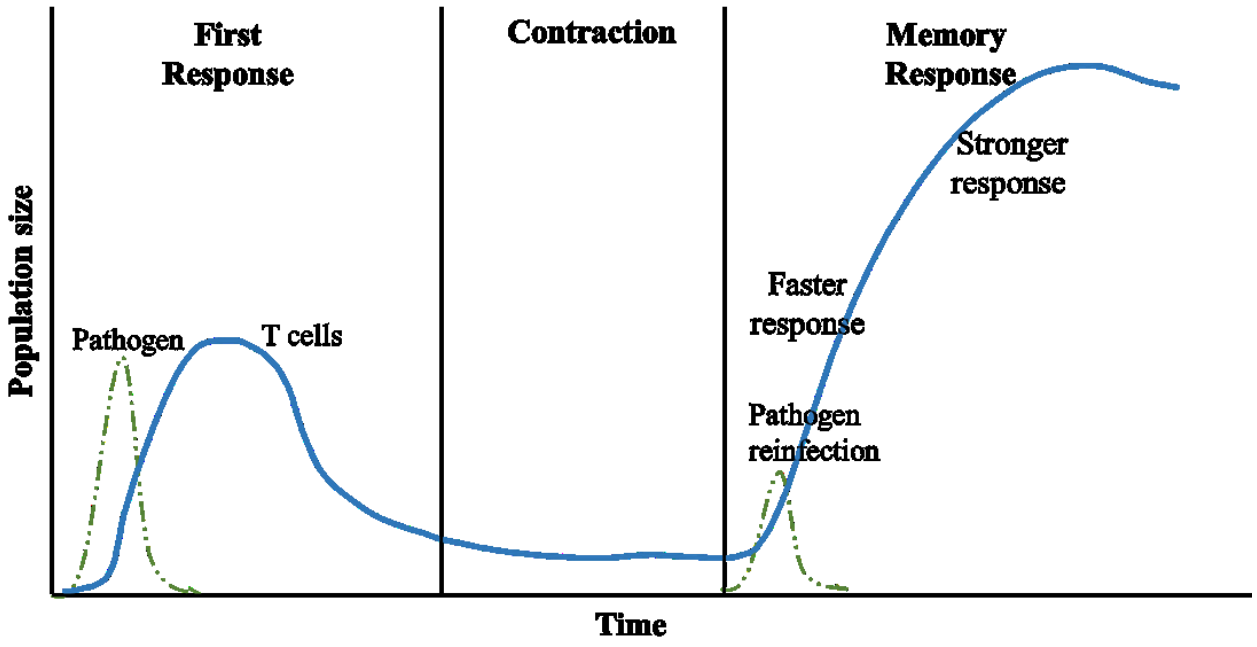

Naïve
CD8
T Cell
CYTOKINE
RECEPTOR
CYTOKINE
IMMUNE
CHECKPOINT
TCR
MHC I
Dendritic
Cell
PATHOGEN

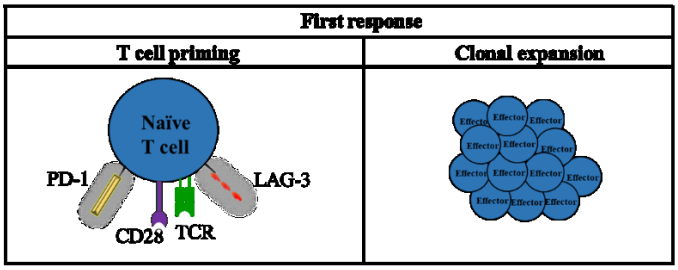
Figure 1.1 Priming of a CD8$^+$ T cell. CD8$^+$ T cells are a pivotal part of the adaptative immune system. After pathogen encounter, naïve T cells differentiate into effector T cells with specialized functions that help to clear invading pathogens. For full naïve CD8$^+$ T cell activation the following four steps are necessary: 1. the engagement of TCR; 2. the recognition of cognate antigen under MHC I context in APC; 3. the engagement of immune checkpoint receptor and ligand; and 4. the interaction with inflammatory cytokines. If this interaction occurs the result is rapid proliferation and differentiation into a more specialized CD8$^+$ T cell.

$CD8^+$ T cells expand and whether or not they successfully eliminate pathogen infection, they return to homeostasis and undergo a rapid systemic reduction during the contraction phase (Fig. 1.2).[33,34] Some studies already have pointed to the fact that T cells are programmed or primed to be memory cells during the very stages of the priming phase.[35] It has been reported that activation and early expansion stages are crucial to determine the fate of $CD8^+$ T cells.[36] In our studies we use recombinant vaccinia virus expressing ovalbumin peptide epitope which is fully immunogenic and elicits a strong $CD8^+$ response[37,38] Vaccinia virus (VV) has been an important tool to study poxivirus infection. It is an important model to study how immune responses are generated during infection.[39–41]

A.


First Response
Contraction
Memory Response
Stronger response
Faster response
Pathogen
T cells
Pathogen reinfection
Population size
Time

B.


First response
T cell priming
Clonal expansion
Naïve
T cell
PD-1
LAG-3
CD28
TCR
Effector

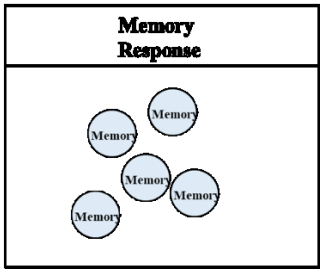

Memory
Response
Memory

Figure 1.2 Kinetics of $CD8^+$ T cell expansion during primary acute response. T cell differentiation during an acute response. (A) Kinetic curve of T cell expansion during a viral infection. (B) After pathogen encounter $CD8^+$ T cells expand once pathogen is cleared, most of those clones die and the population contracts until just few clones survive as memory cells.

Another important aspect in the study of $CD8^+$ T cells is the extensive metabolic changes that happen during activation and memory formation. $CD8^+$ T cells that differentiate into effector cells undergo several changes, in shape and function and metabolic reprograming from mitochondrial oxidative phosphorylation (OXPHOS) to glycolysis.[42]

Numerous studies have reported different metabolic programs during asymmetric $CD8^+$ T cell division in the context of infection response. Once naïve $CD8^+$ T cells are activated they give rise to a daughter and a progenitor cells with opposite metabolic pathways. Naïve and memory cells show a catabolic program while $CD8^+$ T cells that become effector adopt an anabolic metabolism program. [43–51]

Needless to say, during this metabolic changes $CD8^+$ T cells adopt different signaling programs that affect the outcome of their functions. TCF1 is an important transcription factor that determines the fate of a $CD8^+$ T cell becoming a memory cell.[52,53]

Several aspects of $CD8^+$ T cells have been elucidated, however it is still unknown how they form memory. An important motivation to keep deciphering $CD8^+$ T cells memory formation is the design of vaccines. As mentioned in section one, vaccines that stimulate strongly the production of antibodies have been successful. However, such vaccines are not universal for all pathogens like malaria or HIV for example.

There is an undeniable progress in the study of $CD8^+$ T cells. It is important to focus in elucidating the factors that direct $CD8^+$ T cell differentiation that can help modulate and manipulate the outcomes of $CD8^+$ T cell priming. Molecular engineering and pharmacological tools can be used to control $CD8^+$ T cell responses that can be of promising use in the clinic and the development of several therapies including but not limited to cancer treatments and prevention of cancer relapse.

Section 1.3: The making of immunological memories

Immunological memory: an attempt at conceptualization.

As the wild man Endiku helped the great king to learn humility,[2] history and perspective have helped scientist to be humble and to understand how complex the immune system can be. For several years the advancement of what is known as cellular immunology had a slow progress, as Arnold Rich pointed out in 1953 "Literally nothing of importance is known regarding the potentialities of [small lymphocytes], which constituted, "one of the most humiliating and disgraceful gaps in all medical knowledge."[54] It has taken a long time and effort to elucidate what we so far know about immunological memory and $CD8^+$ T cells.

Immunological memory is a unique characteristic of the immune system. Under a classical view, immunological memory is the acquisition of long-term acquired protection through vaccination or infection.[55,56] With a more complex understanding, immunological memory concedes the ability to respond in a faster and more efficient way to the second invasion of a pathogen and constitutes the basis for vaccination.[17]

But what is behind a successful vaccine and the maintenance of immune memory?
We have some effective vaccines that have helped eliminate or minimize the consequences of some mortal and disability causing diseases. The smallpox vaccine was the first vaccine to be used worldwide in humans with great success that in 1979 smallpox was eradicated.[57] To date only smallpox and rinderpest have been

eradicated.[58,59] Without doubt vaccines are one of the greatest medicine achievements. The most effective vaccines nowadays are the ones that stimulate the generation of antibodies.[60] However, there are still diseases that are a challenge for protective vaccines. Among such diseases are Malaria, HIV and Tuberculosis for example, that are resistant to humoral immunity.[61–63] Thus, to target those diseases it is necessary to generate vaccines that aim towards the stimulation of cellular immunity like $CD8^+$ T cells. Such vaccines have proven a major challenge.[63] $CD8^+$ T cell vaccine generation is important because of their specificity to proteins and for granting sterilizing immunity.[64–66] $CD8^+$ T cells are important in the vaccine design; they control intracellular pathogens and also are able to limit tumor establishment and growth.[67,68] $CD8^+$ T cells can also be autoreactive and attack self.[69,70] Therefore, vaccines can also fight cancer and autoimmune diseases and are currently a very active area of research.[17,71–74]

The understanding of immunology and that of immunological memory did not emerge in an orderly form. It also has been slow and challenging to emerge. Discerning the conceptual aspects of immunological memory is still a work in progress, and the mechanisms underlying its formation and orchestration are still evolving. A clear example is the challenging of notion that immunological memory is only limited to B and T cells, when in fact evidence points that exits in a wide set of diverse immune cells like monocytes, macrophages and natural killer cells.[15,56,75–79]

For long the immune system was thought to be a harmless mechanism that protected against external pathogens. But in early 1902 a seminal work by Porter and Richet[†] report an aberrant behavior or the immune system: "anaphylaxis."[80] This important report helps to approach the understanding of immunization with a new perspective. Immunization was not anymore, a way to protect against a pathogen, it was also an event with the capability of causing harmful events and sever disease. Thus, the immune system revels a dark side showing that can harm the host instead of only protecting it. This helps to focus in studying in how immunization works.[81–84]

[†] The thesis author opposes the views of Richet on psychic phenomena or withe supremacy, although his unrelated studies on anaphylaxis are important. The author however would like to point out the importance of Porter in the anaphylaxis studies made with Richet. Porter was a great scientist but forgotten for his humility in declining the Nobel price.

Section 1.4: Creating new memories

Immunology is a science that has a wide array of different concepts, definitions and theories. Several of such concepts are only familiar to immunologists, like "suppressor cells;" and other concepts that are used by non-immunologists such as "monoclonal antibodies."[85] All these concepts emerged to answer questions and to satisfy curiosities. In immunology the understanding and elucidation of some concepts has made slow progress and not without controversies. One example is the case of the understanding of cellular immunology. [86] In fact one of the longest battles in immunology, has to do with humoral vs cellular immunology.[87]

Parallel to satisfying the curiosity around immunity, new doubts have emerged. Nowadays a controversial topic in the immunology field surrounds immunological memory. For example the understanding of how T cell memory is successfully generated is a current and active field of research.[16] $CD8^+$ T cells play a pivotal role in cellular immunology, and their study is important for vaccine design.[88]

Although studies have described how T cell memory is generated following acute infection, less is known about the maintenance of T cell memory in the context of immune inhibitory receptors.

The formation of $CD8^+$ T cell memory is an active research topic, understanding how it happens can help towards the design of effective vaccines. Understanding how $CD8^+$ T cells go from naïve to effector to memory and orchestrate metabolic changes that influence and imprint their future behavior as memory cells.[73]

$CD8^+$ T cell memory

After pathogen clearance, some effector T cells that responded to infection persist and become memory T cells.[27] There are several proposed models as how $CD8^+$ T cell memory is formed.[15,16,36,89,90] In our studies we make use of parameters proposed for different memory models. It has been proposed that a small fraction of effector $CD8^+$ T cells survive the contraction phase and are identified as memory precursor effector cells (MPECs) and can be distinguished because of their high expression levels of CD127 and decreased expression of KLRG receptor. The other dominant population of effector $CD8^+$ T cells has high expression of KLRG1 and low expression of CD127 and it has been identified as short-lived effector cells (SLECs).[91–95] These two populations have been observed in different infectious contexts in mouse and human, however the presence of MPECs and SLECs is not the only criterion for identifying memory precursors after an immune response; nor are they universally required for the formation of $CD8^+$ T cell memory.[96–98]

The "asymmetric cell fate" models shows that a single precursor T cell can yield both an effector and a memory T cell through asymmetric division.[52,99–102] In our studies we work under the parameter of early priming and asymmetric cell differentiation.

Other models propose the influence of other factors in the fate of a $CD8^+$ T cell, for example TCR signaling strength that has been negatively associated with memory formation.[103,104] Additionally, the transcription factor profile becomes distinctive for T cells that become effectors when compared to T cells that become memory. IRF4 for example has been shown to be important in the formation and expansion of effector cells.[105] Other crucial transcription factors reported for $CD8^+$ memory formation are Eomes and TCF1.[106,107]

Additionally, TCF1 is also highly expressed in naïve $CD8^+$ T cells, promoting self-renewal. TCF1 is downregulated in effector cells and is expressed again in memory cells. Expression of TCF1 is important for long-term survival and homeostatic proliferation.[52,108]

Section 1.5: $CD8^+$ T cells in the tumor microenvironment

Immunotherapy

Our curiosity of how the immune system works has not been satisfied; new concepts and questions emerge the more we learn. But we not only want to learn more about the functioning of the immune system but also how to use it and hack it to our benefit. For example, with immunotherapy, in specific cancer immune checkpoint therapy.

Nowadays the idea that the immune system plays a pivotal role in conferring protection against cancer is well established. The first concept of immunotherapy can be attributed to Dr. William Coley's efforts in 1893 to treat tumors through a local injection of " Coley's Toxins," a mixture of live bacteria used as an immune stimulant.[109,110]

By 1890 there was already the concept of immune surveillance that was proposed by Ehrlich: a scanning of the human body the immune system to eradicate transformed cells. [111] At the beginning of the 20th century the notion of "immunosurveillance" of cancer was proposed. Similarly, by the early decade of the 70's Burnet and Prehm brought back the concept of tumor immunosurveillance. They purposed that tumors were eliminated by the immune system before they became clinically detectable.[112,113]

A.

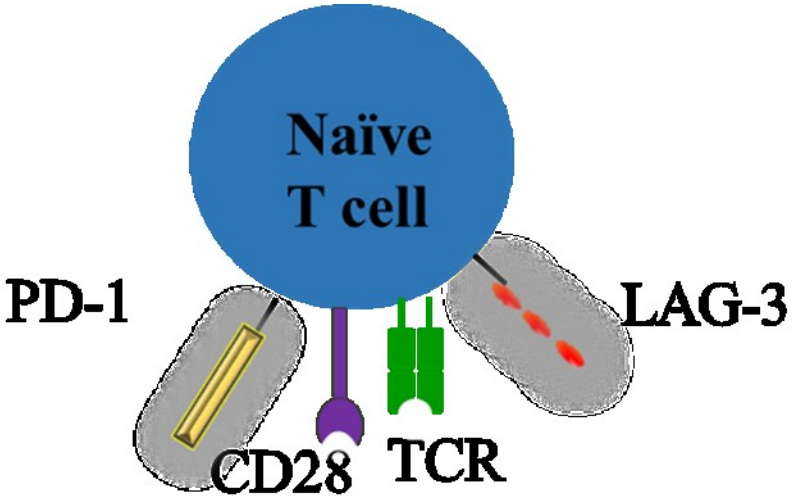


Figure 1.3 Immune checkpoints. Immune checkpoints contribute to regulate the amplitude and quality of T cell responses. Immune checkpoints can stimulate a $CD8^+$ T cell response namely CD28 or can inhibit responses. Programmed cell death 1 (PD-1) and lymphocyte-activation gene 3 (LAG-3) are inhibitory immune checkpoints that contribute to T cell homeostasis, activation and differentiation.

But why immune checkpoint therapy?

Immune responses are potent and need to be tightly regulated to avoid unnecessary or unwanted responses that could result in tissue damage and the break of self-tolerance.[114–116] Immune checkpoints are a wide array of molecules expressed in the surface of cells that function as co-receptors that either promote or inhibit T-cell activation (Fig.1.3).[117] During T cell activation inhibitory receptors are up regulated as well as stimulatory ones. This is a way to regulate overstimulation of the immune cells responding to an infection after pathogen encounter.[118] Kummel and Allison described the immune checkpoint cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)'s ability to inhibit T cell responses and how using a blocking antibody against CTLA-4 could promote T cell mediated tumor rejection.[119] Several studies followed and immune checkpoints have been the focus of research since, their understanding and elucidation has contributed to the development of blocking antibody based therapies that restore $CD8^+$ T cell function and tumor immunity.[109]

Tumor infiltrating $CD8^+$ T cells' functions can be modulated by using antibodies that target immune check points that either stimulate or inhibit cell functions.[120] This modulation of cells can be achieved by combining the blocking of several checkpoints.[121,122]

Immune check points

Immune checkpoints contribute to regulate the amplitude and quality of T cell responses and have been a major focus of study to enhance current immune therapies.[123] Programmed cell death 1 (PD-1) and lymphocyte-activation gene 3 (LAG-3) are immune checkpoints that contribute to T cell homeostasis, activation and differentiation.[89,124–128] Previous studies in murine tumor models report enhanced T cell function in the absence of PD-1 and LAG-3 as a result of the synergy between these inhibitory pathways.[129] The effect of combined loss of PD-1 and LAG-3 on the formation of long-term $CD8^+$ T cell memory, however, has not been evaluated.

PD-1

Immunotherapy for cancer is currently used with efficacy and promising results in the clinic. However more studies are needed since not all patients benefit from PD-1 therapy.[130] A common way to therapeutically target PD-1 in the clinic is through the use of antibody blockade of PD-1/PDL-1 to disrupt the ligand/ligand interaction.[131]

PD-1 was cloned in 1992; it was first identified as a type I transmembrane protein.[132] Currently it is known as an immunoreceptor tyrosine-based switch motive (ITSM) containing inhibitory molecule.[124] PD-1 is a member of the B7 family of co-stimulatory/co-inhibitory receptors and after initial immune stimulus is transiently expressed in several immune cells: $CD4^+$ and $CD8^+$ T cells, B cells and natural killer (NK) cells, macrophages, dendritic cells and some cancer cells.[67,133–140] It has two known ligands: PDL-1that is expressed ubiquitously and PDL-2 that has restricted expression

when compared to PDL-1, in that is expressed in APC and macrophages.[128] PDL-1- has been reported to be expressed in some tumor environments.[141]

PD-1/PDL-1 interactions are antagonists of CD80/CD28 co-stimulation such interaction results in diminished to cytokine production, cell arrest and stops production of the pro-survival Bcl-X.[142,143] PD-1 strongly interferes with TCR signal transduction, even at low expression, by recruiting Src homology region 2 domain-containing phosphatase-1 (SHP1) and protein-tyrosine phosphatase 1D (SHP2) that interfere with the CD28 phosphorylation and interaction with TCR.[124]

PD-1 mediates central and peripheral immune tolerance in addition of immune exhaustion.[144–147] PD-1 has been reported to potentially help metabolic reprograming of T cells from effector to long-lived memory cells. This is seen by the shift from high glycolysis towards fatty acid B-oxidation.[46,148]

PD-1 is also a maker of exhausted T cells.[149] High expression of PD-1 has been linked to cell exhaustion in chronic viral infections.[150] When a $CD8^+$ T cell is exhausted, it becomes faulty in secreting cytokines, it stops proliferating and is unable to accomplish tolerance related functions.[151–153] Tumor infiltrating T cells have a high expression of PD-1 in some tumor microenvironments and PDL-1 signaling is how tumors can avoid destruction by cells expressing PD-1 like CD-8^+ T cells for example.[122,154–158] Through the blocking of PD-1 signaling anti-tumor response can be achieved.[159,160] The tumor microenvironment is potentially hosting several immune cells like $PD1^+$ T cells that are

antigen experienced but are exhausted or inactivated by the tumor microenvironment.[161,162] The molecular elucidation of how PD-1 blockade therapy works has not been reported yet. However, it is a promising hope for the treatment of cancer.

LAG-3

Lymphocyte activation gene 3 (LAG-3) is also a co-inhibitory immune checkpoint that plays an important role in T-cell tolerance and T cell modulation, impeding endogenous unnecessary immune responses in non-aberrant physiological conditions.[163,164] As in several other checkpoints, LAG-3 is highly expressed in the tumor microenvironment giving advantage to the tumor and allowing evasion of anti-tumor immunity.[165]

LAG-3 was cloned in 1990 and it was identified as a membrane protein.[166] Structurally LAG-3 is similar to the $CD4^+$ molecule. It is considered an activation marker for $CD4^+$ and $CD8^+$ T cells and it is also expressed on NK cells, B cells, Tregs and DCs.[129,167] LAG-3 expression is transient and short lived at the protein level in the early stages of T cell activation. LAG-3 is highly expressed by T-regs mostly at the mRNA level compared to the protein level.[168] Studies have shown that the primary function of LAG-3 is to inhibit T cell expansion as well as to control the size of the memory T cell pool.[89] Additionally it regulates T cell homeostasis and plays a key role enhancing the functions of regulatory T cells.[169] LAG-3 has been shown to interact with MHC-II with higher affinity than $CD4^+$ T cells.[163,170] Therapeutically, blocking the interaction of LAG-3 and MHC-I expressed by APC has shown an increase of infiltrating $CD8^+$ T cells in the tumor microenvironment of murine models of renal cancer and melanoma.[171]

Studies have reported that LAG-3 potentially associated with TCR/CD3 complexes on T cells results in a crosslinking that stops the calcium response to CD3 stimulation.[172] How the downstream signaling works is still being elucidated. Exhausted $CD8^+$ T cells have also been reported to highly express LAG-3 conjointly with PD-1. [154] LAG-3 KO mice do not however, develop spontaneous lymphoproliferative disease. Such mice live for about a year and develop dermatitis and die usually of hard to cure skin infections. In the clinic antibody against LAG-3 binds to tumor infiltrating $CD8^+$ T cells and helps reduce tumor burden by activating antigen specific T cells.[173]

PD-1 and LAG-3 in CD8+ memory formation.

But why the interest in the formation of $CD8^+$ T cell memory in the context of inhibitory receptors? Several studies have shown the importance of the PD-1 and LAG-3 receptors in regulating T cell activation and function in a synergistic manner in mouse tumor models.[129,174] Furthermore, it has been reported that there is a reduction of tumor burden in the absence of PD-1 and LAG-3.[129] However, other work has shown that loss of PD-1 signaling in T cells in a chronic infection model contributes to an exhausted-like state for memory cells. Studies show that the absence of LAG-3 in T cells increases clonotypic expansion and effector function in a tumor model.[126]

The combined effect of the loss of PD-1 and LAG-3 on the formation of long-term memory has not been described. Combinatorial immunotherapies have proven to be a

powerful approach to treat cancer. Immunotherapy directed toward PD-1 and LAG-3 has shown promising results in multiple trials. Understanding how PD-1 and LAG-3 interact will help to improve current therapies to reduce tumor burden and also contribute to the development of durable and preventive therapies. One of the main goals of immunotherapy is to develop lasting immunity to cancer that can reduce recurrence. Understanding the formation of long-term T cell memory in the context of immune checkpoint blockade can improve cancer immunotherapy design.

CHAPTER II: METHODS

Mice and infections

All mice were housed and used in accordance with the guidelines of the Johns Hopkins University Institutional Animal Care and Use Committee under an approved protocol. CD45.1 mice were purchased from Charles River. OT-1 mice were originally obtained from Dr. M. Bevan (University of Washington), and bred onto a RAG2 -/- background at JHU prior to use. PD-1 KO mice on a C57/B6 background were provided by Dr. Lienping Chen (Yale University) with permission from Dr. T. Honjo (Kyoto University). LAG-3 KO mice on a C57BL/6 background were provided by Dr. Yueh-Hsiu Chien (Stanford University, CA) with permission from Dr. Christophe Benoist and Dr. Diane Mathis (Joslin Diabetes Center, Boston, MA).

P14 TCR transgenic mice recognizing LCMV peptide gp33-41/H2D^b were purchased from The Jackson Laboratory (Bar Harbor, ME). For experiments modeling the acute response to primary infection, mice were infected with 10^6 plaque-forming units (PFUs) of Vaccinia-OVA (VV-OVA) or 10^7 colony-forming units (CFU) of Listeria-ova (LM-OVA). For re-challenge experiments assessing recall responses, mice were first infected with VV-OVA and subsequently re-challenged at day 60 post primary infection with 10^7 CFU attenuated Listeria, a gift of Dr. Pete Lauer (Aduro Biotech). VV-OVA was gift from Dr. Drew Pardoll, it was originally generated by Dr. Nick Restifo by homologous recombination into the WR strain of Vaccinia. It encodes full length ovalbumin. To generate acutely infections in P14 mice we used $2x10^5$ PFU of LCMV, Armstrong strain.

Adoptive Transfer and lymphocyte isolation

$CD8^+$ T-cells isolated from the spleen and peripheral lymph nodes of 4 - 6-week-old OT-1 RAG2 KO mice. $CD8^+$ T cells were enriched from the spleen using Naive $CD8^+$ T cell positive isolation kit (Miltenyi), according to manufacturer's protocol. 5x10^5 $CD8^+$ T cells were then administered by tail vein injection into congenically distinct recipient mice (C57/B6 CD45.1) one day prior to infection with VV-OVA. At day 8 p.i. Spleens, livers, lungs and LN were harvested at day 8 post infection (p.i.) for the primary infection and at day 64 for secondary infection. The phenotype of adoptively transferred cells was determined by flow cytometry. Same procedures were followed for PD-1 KO, LAG-3 KO, DKO $CD8^+$ OT-1 and P14 T-cells isolation. To assess maintenance and/or rejection of transferred cells, organs were harvested at day 21 p.i (Fig 2.1).

In vitro stimulation and TCF1 staining

To model the process whereby $TCF1^{hi}$ activated CD8+ T cell progenitors of a defined specificity give rise to irreversibly determined $TCF1^{lo}$ effector cells while self-renewing $TCF1^{hi}$ progenitors, as evident during acute infection.[52] TCR transgenic T cells were activated in vitro.[43,52,175]

Whole spleens from OT-1 RAG2 KO mice and P14 mice were processed as described previously,[125] they were labeled with cell proliferation dye (CTV), and incubated and stimulated with peptide SIINFEKL or LCMV gp33 peptide KAVYNFATM for 4 days in RPMI complete media. Some groups received 50 ug/mL of either αPD-1 (clone J43) and/or αLAG-3 (clone C9B7W), or rat IgG1 and Armenian Hamster IgG isotype control

antibody (Bioxcell, West Lebanon, NH). Some groups were treated with N-acetyl cysteine (Sigma, 7mM) or Pictilisib (Class I PI3K inhibitor, Selleckchem, 1μM). In day 4 of incubation cells were collected and stained for TCF1 as previously described.[176]

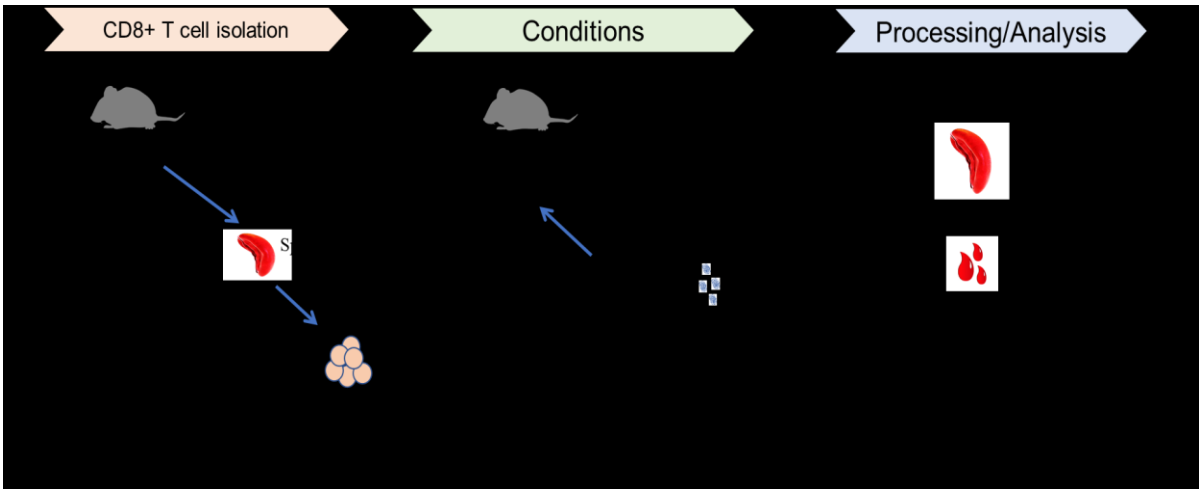
Flow cytometry

To discriminate live from dead cells, all cells were stained with LIVE/DEAD Fixable Dead Cell Stain (Life Technologies). Surface staining was performed as described previously[125]. Anti- CD8+α (5H10) was purchased from Invitrogen. The following antibodies were purchased from Biolegend: CD45.2 (104), IFNγ (XMG1.2). LAG-3 (C9B7W), TNFα (MP6-XT22), Granzyme B PD-1 (J43), T-bet (ebio4B10), were purchased from Ebioscience. All antibodies were used according to manufacturer's protocol. Intracellular cytokine staining was performed following a 5 hour incubation at 37°C in 5% CO2 in complete media supplemented with the 50μM of OVA-SIINFEKL and protein transport inhibitor (Ebioscience). Cells were then fixed and stained using Cytofix/Cytoperm kit (BD) according to the manufacturer's instructions. Flow cytometry was performed on a FACsCalibur or LSR II (BD, San Diego, CA), and analyzed using FlowJo software (Tree Star). For intracellular staining of transcription factors, a FoxP3 staining kit (eBioscience) was used according to the manufacturer's instructions.

Data Analysis and Statistics

All data were generated using FlowJo software (Treestar), Graphpad Prism software, version 5.0c (GraphPad).

A.


CD8+ T cell isolation
Conditions
Processing/Analysis

B.

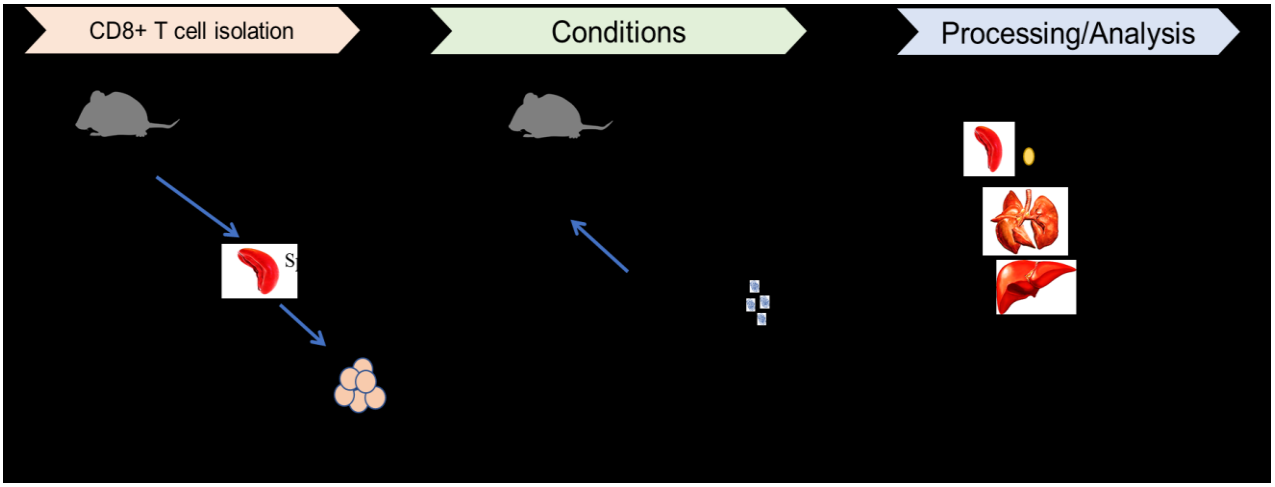
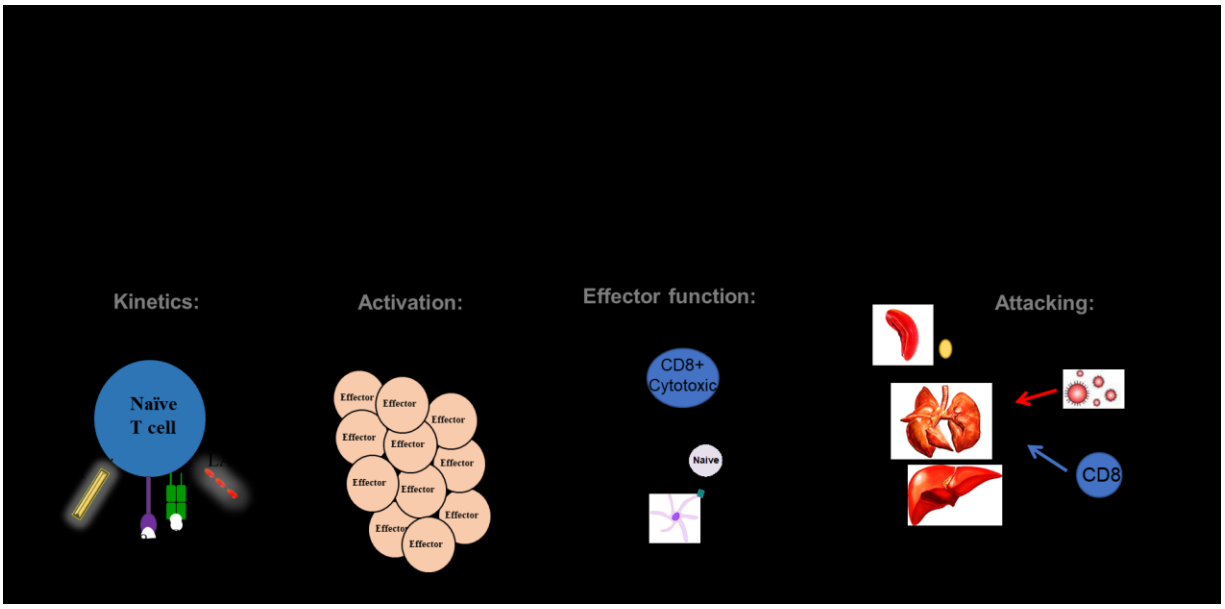

CD8+ T cell isolation
Conditions
Processing/Analysis

Fig 2.1. Mouse models and adoptive transfer schema. Graphical description of the two types of conditions used with OT-I mice. $CD8^+$ T cells were isolated from a CD41.2 donor mouse and adoptively transferred to a CD45.1 host mouse. (A) In one condition, the mice either received blocking antibody and the spleen and blood where collected to read their phenotype in the flocytometer. (B) Cells with different knocked out molecules were used in stead of the blocking antibody and the spleen, liver, lymph nodes and lungs were collected to read their phenotype in the flocytometer.


Kinetics:
Activation:
Effector function:
Attacking:
Naïve
T cell
Effector
CD8+
Cytotoxic
Naive
CD8

Chapter III: Expansion/kinetics

Section 3.1: Introduction

After the exposure to a foreign antigen through either infection or vaccination, the immune system elicits a strong response to protect the host.[177] During the acute phase of infection cells need to be primed, expand, become effectors, traffic to sites of infection and carry out pathogen clearance.[18] $CD8^+$ T cells, one of the main mediators of the adaptative immune response, are one of the key players of the adaptive immune system.[178] Their activation is necessary for the control of pathogen invasion.[90] One of the primary goals of $CD8^+$ T cell response to infection is to generate large numbers or clones to help eliminate infection.[179]

It is estimated that there is 1 naïve $CD8^+$ T cell in 10,000, specific for any particular antigen.[24] Once activated a naïve $CD8^+$ T cell can dramatically expand to 50,000 fold.[180] Because of such dramatic expansion and a powerful response of the immune system there are several mechanisms in place to prevent immune reactions to self-proteins.[181] A naïve $CD8^+$ T cell must receive 3 signals to engage in an immune response: the recognition of peptide in the context of MHC class I, signaling through the engagement of costimulatory receptors and cytokine signaling.[29,182,183]

There are several factors that enhance the magnitude of the immune response: frequency of naïve $CD8^+$ T cells, number of naïve $CD8^+$ T cells recruited to site of infection,

duration and quality of infection, immunodominance and peptide affinity for example.[184–186] But what exactly directs the size of the clonal pool of $CD8^+$ T cells has been determined with precision to be the precursor frequency of naïve $CD8^+$ T cells for a specific antigen. In other words, the number of $CD8^+$ T cell specific for an antigen before infection, is the critical parameter defining the magnitude of expansion.[187,188]

Naïve $CD8^+$T cells scan between 160 and 300 dendritic cells per hour and stay in a lymph node for about half a day to a day.[189] When a naïve $CD8^+$ T cell encounters a dendritic cell with a cognate antigen, they stay in contact for about a day during which they "blast."[190–193] Short exposure to cognate antigen - as little as 2 hours- is enough to drive a naïve $CD8^+$ T cell to several rounds of division in vitro.[194,195] However in vivo a period of 20 hours is necessary to fully activate a naïve $CD8^+$ T cell that afterwards can differentiate into effector cells and later into memory cells[35,47,195–197]

In the next several hours to days after exposure $CD8^+$ T cells enter a profound change: their interaction with dendritic cells causes them to increase in size by doubling their protein production and content, they change their metabolism and increase their RNA content about 30 fold to 1,300 mRNAs.[42,46,198,199] Naïve $CD8^+$ T cells leave their inactive resting state and become metabolically dependent on glucose to satisfy their need for rapid proliferation and biosynthesis to ensure growth and differentiation.[46] All of these changes ensure a naïve $CD8^+$ T cell is ready to proliferate.

During the acute phase of our studies we use VV-OVA that causes proliferation of OT-I cells initially in the lymph nodes and spleens.[38] $CD8^+$ T cells respond to VV-OVA by dividing and inducing cytolytic activity and production of IFN-γ, and TNF-α.[200,201] Once naïve $CD8^+$ T cells are primed they expand and reach a peak at about 7 to 10 days after infection; the exact day of peaking is pathogen dependent.[18,26,36,202] Once peak of expansion is reached, the proliferative potential of the differentiated $CD8^+$ T cells declines and they die by apoptosis.[29]

The rapid proliferation and expansion of immune cells during an immune response is so powerful that to inhibit, modulate and control unwanted responses the immune system has in place several tightly regulated mechanisms .[203,204] Inhibitory receptors are in part responsible for regulating uncontrolled or unwanted expansion of activated T cells.[205,206] T cells express counterparts of stimulatory molecules known as inhibitory check points. They help to modulate the T cell response to pathogen infection.[115]

The co-inhibitory receptor LAG-3, has been reported to regulate T-cell function and to control effector T-cell expansion and homeostasis.[89,127,168]Adittionally, other studies show that PD-1 and LAG-3 inhibitory co-receptors act synergistically[207], and both enhance $CD8^+$ T cell clonal expansion,[129] as observed in tumor bearing mouse models and autoimmune mice models.[129,174]

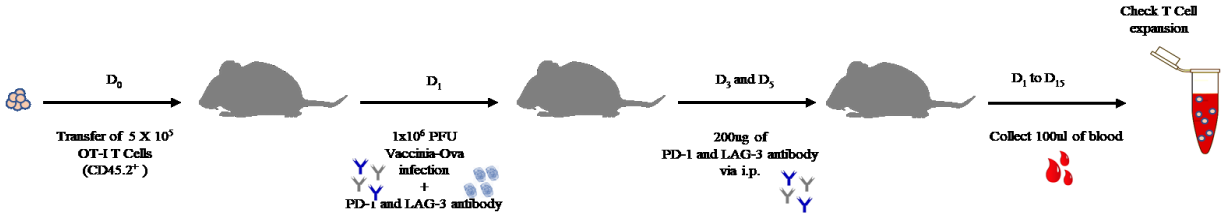
In our studies we outline the kinetics of $CD8^+$ T cell immune response in the absence of PD-1 and LAG-3 during the primary response to acute infection of VV-OVA. Previous studies have reported that in the spleen $CD8^+$ response to VV-OVA is comparable to that induced by lymphocyte choriomeningitis virus (LCMV).[208]

Section 3.2: Results

Loss of PD-1 and LAG-3 increases effector cell expansion in the peripheral blood during primary acute infection of VV-OVA.

To determine the role of the inhibitory receptors PD-1 and LAG-3 in $CD8^+$ T cell differentiation, we used an OT-I PD-1/LAG-3 double KO mouse model. After immunization with VV-OVA, $CD8^+$ T cells are considerably increased in number in circulating blood in the spleen. We also observed increased number in the liver, lymph nodes and lung. We first examined the early expansion of $CD8^+$ T cells by assessing their presence in circulating blood (Fig. 3.1B). In the absence of PD-1 and LAG-3, $CD8^+$ T cells had proliferative advantage; their presence in the circulating blood was noticeable earlier than the wild type (WT) group. The absence of both inhibitory receptors results in a steep and wide kinetic curve when compared to the WT counterpart.

A.


Check T Cell expansion
D0
Transfer of 5 X 10^5 OT-I T Cells (CD45.2+)
D1
1x10^6 PFU Vaccinia-Ova infection + PD-1 and LAG-3 antibody
D3 and D5
200ug of PD-1 and LAG-3 antibody via i.p.
D1 to D15
Collect 100ul of blood

B.

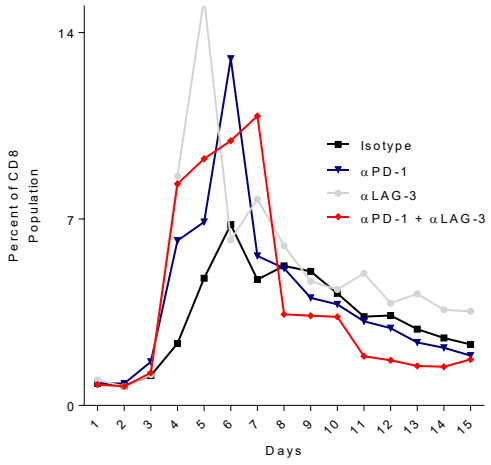
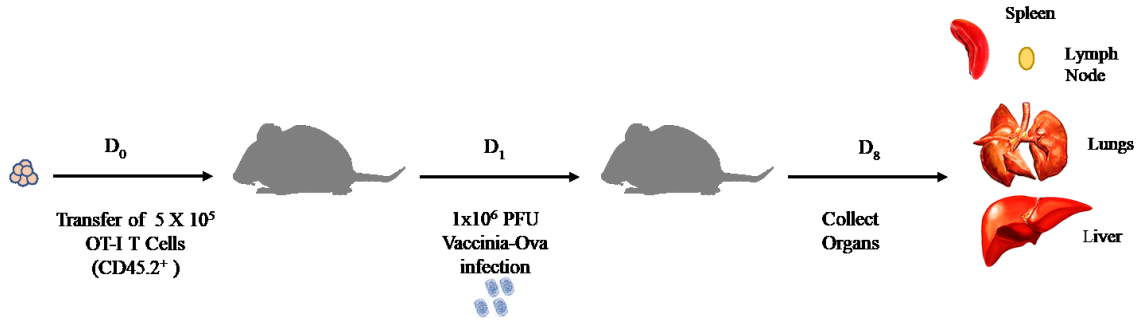

Percent of CD8 Population
Days
Isotype
αPD-1
αLAG-3
αPD-1 + αLAG-3
0
7
14
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 3.1 Absence of PD-1 and LAG-3 influences $CD8^+$ T cell expansion. (A) Schematic representation of blood collection to check clonal expansion of adoptively transferred $CD8^+$ T cells. Blood was collected daily from the tail. (B) Kinetic curve of $CD8^+$ T cell expansion showing in red the early and wider clonal of cells with double blockade when compared to the wild type.

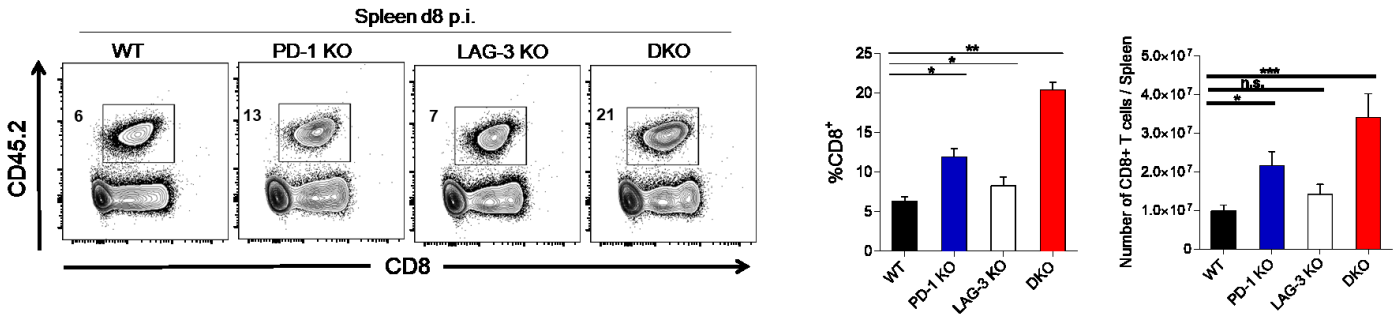
Loss of PD-1 and LAG-3 enhances effector cell expansion in lymphatic and systemic organs during primary acute infection of VV-OVA.

Once we learned that in the absence of PD-1 and LAG-3 $CD8^+$ T cells expanded during the early days of primary phase of the acute infection, we investigated the proliferating capacity of OT-I $CD8^+$ T cells during the acute response in the systemic and lymphoid organs at the peak of the expansion. We sought to test the hypothesis that in a murine tumor-free model the absence of PD-1 and LAG3 influences cell expansion. At day 8 post infection (p.i.) mice organs were collected (spleen, liver, lymph nodes and lung), and when compared to WT, PD-1 KO and LAG-3 KO double knock out (DKO) $CD8^+$ T cells (PD-1 KO/LAG-3 KO) have greater expansion as shown by the presence of a greater percent and absolute number of cells in the spleen, liver, lung and lymph nodes when compared to wild type (Fig. 3.2A - 3.2E).

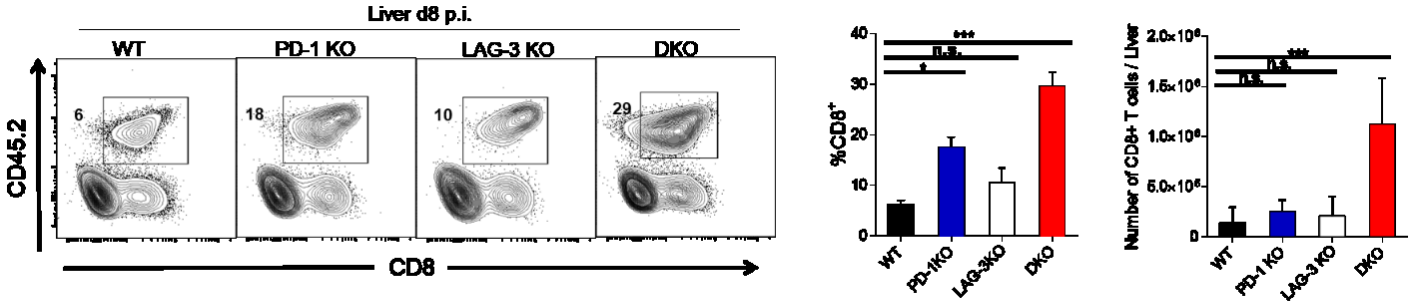
A.


Spleen
Lymph Node
Lungs
Liver
D0
D1
D8
Transfer of 5 X 10^5 OT-I T Cells (CD45.2+)
1x10^6 PFU Vaccinia-Ova infection
Collect Organs

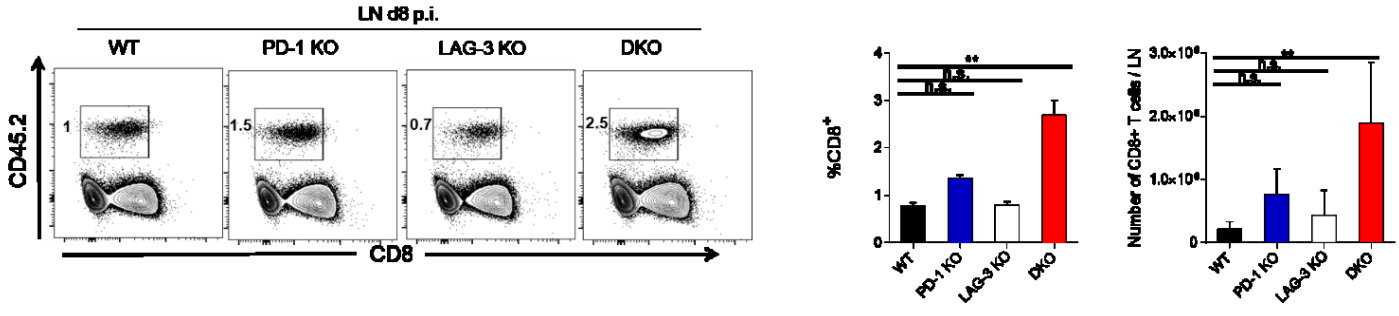
B.


Spleen d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
CD45.2
CD8
6
13
7
21
%CD8+
Number of CD8+ T cells / Spleen
n.s.

C.


Liver d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
CD45.2
CD8
6
18
10
29
%CD8+
Number of CD8+ T cells / Liver
n.s.
*
***
**
WT
PD-1KO
LAG-3KO
DKO

D.


LN d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
CD45.2
CD8
1
1.5
0.7
2.5
%CD8+
Number of CD8+ T cells / LN
n.s.
**
WT
PD-1 KO
LAG-3 KO
DKO

E.

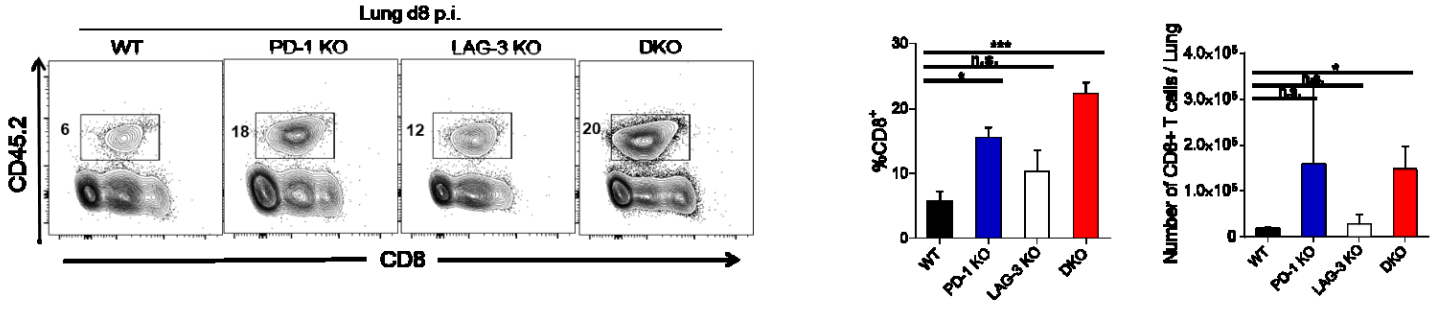

Lung d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
CD45.2
CD8
6
18
12
20
%CD8+
Number of CD8+ T cells / Lung
WT
PD-1 KO
LAG-3 KO
DKO

Figure 3.2 PD-1/LAG-3 absence influences $CD8^+$ T cell expansion in the acute phase of infection. (A) Schematic representation of organ collection. (B - E) Frequency and absolute numbers of transferred WT, PD-1 KO, LAG-3 KO and DKO T cells in spleen, liver, lymph node and lungs at day 8 p.i. N = 5 mice / group. Experiments were repeated twice. *$p<0.05$. **$p<0.001$, Kruskal-Wallis test with Dunn's correction for multiple comparisons.

Section 3.3: Discussion

For an effective response to infection, effector $CD8^+$ T cells need to expand and respond to the invading pathogen. $CD8^+$ T cells acquire cytolytic functions and secrete cytokines. The first step during the response to infection, is the recognition of cognate antigen by $CD8^+$ T cells; in our studies we use vaccinia ova engineered to express ova peptide. When CD8+ T cells respond to infection, they need to pass through an initial activation and an expansion phase. Virus-specific $CD8^+$ T cells, in our studies OT-I, respond to viral infection by sustaining several rounds of cell division that results in clonal expansion.

Vaccinia virus generates immunity through the interaction of dendritic cells that are the best activators of $CD8^+$ T cells.[209] Transferred OT-I cells first proliferate in response to antigen in the spleens and draining lymph nodes; after day 2 several rounds of division have already happened.[38] Previous experiments using murine tumor models have shown that in the absence of PD-1 and LAG-3, $CD8^+$ T cells increase in number and effector function.[129] Here we observe the expansion of $CD8^+$ T cells in the absence of PD-1 and LAG-3 in tumor and autoimmunity free mice during an acute infection with vaccinia-ova. We sought to test the hypothesis that in a murine tumor-free model the absence of PD-1 and LAG-3 influences cell expansion.

First, we tested the ability of $CD8^+$ T cells to expand in the absence of PD-1 and LAG-3 during the primary response of acute infection. We observed the cells in circulating blood from very early stages of clonal expansion to the start of the contraction phase. We report the kinetics of the $CD8^+$ T cells in the absence of PD-1 and LAG-3 cells and whether the peak of the response varies from the wild type cells. By day 3 we were able to quantify $CD8^+$ T cells in the circulating blood, consistent with previous reports that $CD8^+$ clonal expansion starts at day 2 in the spleen.[210] The peak of clonal expansion in response to VV-OVA is at day 5.[210] Consistent with the kinetics of VV-OVA, in our studies the wild type group showed peak at day 5. We observed that in the absence of PD-1 and LAG-3 $CD8^+$ T cells initiate expansion earlier when compared to WT in the circulating blood. Additionally, they sustain an amplified response being at the peak of the response before and at the same time than WT cells do. Both groups contract at a similar time point. In conclusion the absence of both inhibitory receptors results in a steep and wide kinetic curve when compared to the WT counterpart. (Fig. 3.1A, 3.2B).

Once we confirmed that our cells expanded early after acute infection with VV-OVA, we tested the hypothesis that DKO cells have an enhanced expansion also in systemic and lymphatic organs.

Consistent with past results and the negative regulatory role of the PD-1 pathway, the absence of PD-1 enhanced proliferation of $CD8^+$ T cells. [211] The DKO cells also behaved as expected but in a tumor free environment, in a synergistic form with the absence of LAG-3 not affecting but enhancing clonal expansion. [129] In our model $CD8^+$ T cells were

able to receive and process the needed signals to identify antigen, activate and expand. With these results we show that the absence of PD-1 and LAG-3 contributes to a greater clonal expansion of $CD8^+$ T cells in a tumor free model during the acute phase of infection. We show that the absence of PD-1 and LAG-3 enhances $CD8^+$ T cell expansion since early points after acute infection with VV-OVA.

As expected after pathogenic stimulation the DKO cells were able to expand and generate clones. But not only were able to expand but did so in greater numbers when compared with the wild type.

Chapter IV: Effector function

Section 4.1: Introduction

Previous studies have reported that at the peak of clonal expansion two subsets of differentiated $CD8^+$ T cells are distinguished: KLRG-1^{hi}/$CD127^{lo}$ and KLRG-1^{lo}/$CD127^{hi}$.[94] T-bet directs the formation of KLRG-1^{hi}/$CD127^{lo}$ effector $CD8^+$ T cells, and KLRG-1 has been used as a marker of differentiation.[94,212] Other studies show that the expression of KLRG-1 and the loss of TCF1 give rise to terminally differentiated effector T cells, a topic that we will discuss in depth in future chapters.[43]

Additionally, evidence supports that T-bet and Eomesodermin (Eomes) are highly important in the formation of optimal performance of effector and memory $CD8^+$ T cells.[179] During the priming phase, T-bet and Eomes induce expression of IFN-γ, granzyme B, perforin, CXCR3 and CXCR4.[179] In the absence of both T-bet and Eomes there is aberrant production of IL-17 that results in excessive infiltration of neutrophils and lethal inflammatory syndrome.[213]

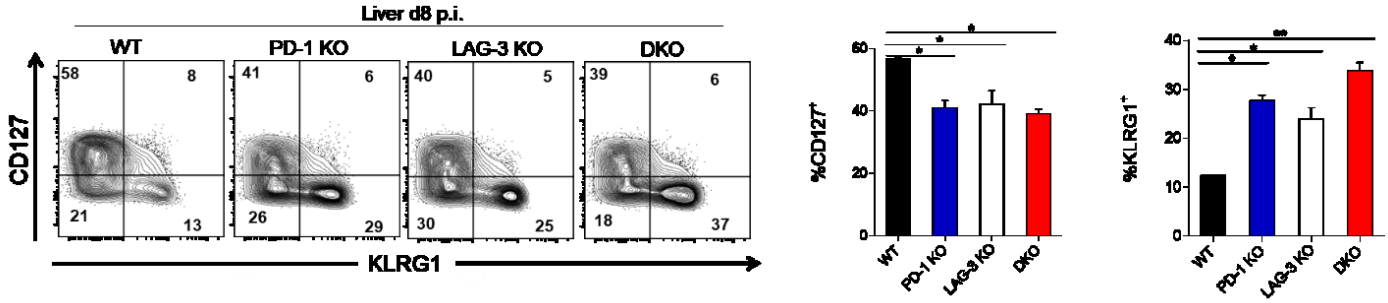
T-bet has also been linked to the regulation of expression of effector functions while Eomes is involved in directing the expression of proteins to maintain the T cell memory pool.[94,106,107,214] It is important to mention too that the stability, functionality and differentiation of $CD8^+$ T cells seems in part to be commanded by the expression ratio

between T-bet or Eomes.[179] Wherry and colleagues have reported that when cells with an exhausted profile is associated with an inverse relationship between T-bet and Eomes[215]

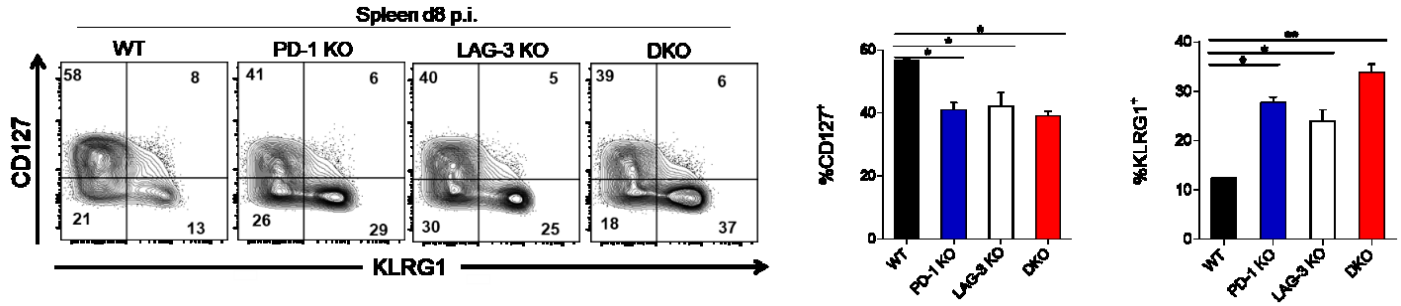
Section 4.2: Results

To determine the role of inhibitory receptors in $CD8^+$ T cell memory formation, we used an OT-I PD-1 KO/LAG-3 KO mouse model. As we reported in chapter III, at day 8 (p.i.), single knockout (PD-1 KO or LAG-3 KO) as well as DKO OT-I cells showed greater clonal expansion compared to wild type (WT) (Fig.4.2 B-E). Additionally to enhanced expansion we identified two subsets of $CD8^+$ T cells previously reported.[216] We found that KLRG-1^{hi}/$CD127^{lo}$ expression is increased in DKO $CD8^+$ T cells. A larger percentage of DKO $CD8^+$ T cells were $KLRG1^{hi}$/$CD127^{lo}$ compared to WT or single KO cells (Fig. 4.1C), suggesting that DKO T cells have an increased percent of differentiated effector cells.[44]

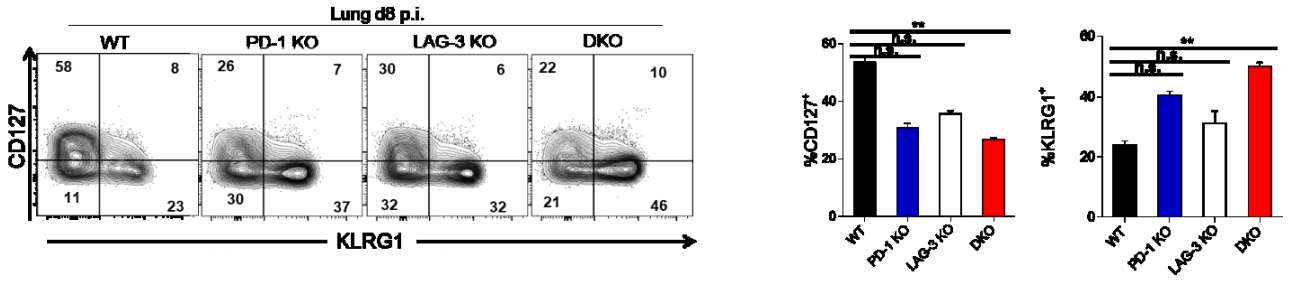
A.


Liver d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
CD127
KLRG1
58
8
21
13
41
6
26
29
40
5
30
25
39
6
18
37
%CD127+
%KLRG1+
WT
PD-1 KO
LAG-3 KO
DKO

B.


Spleen d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
CD127
KLRG1
58
8
21
13
41
6
26
29
40
5
30
25
39
6
18
37
%CD127+
%KLRG1+
WT
PD-1 KO
LAG-3 KO
DKO

C.


Lung d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
CD127
KLRG1
58
8
11
23
26
7
30
37
30
6
32
32
22
10
21
46
%CD127+
%KLRG1+
n.s.
**

D.

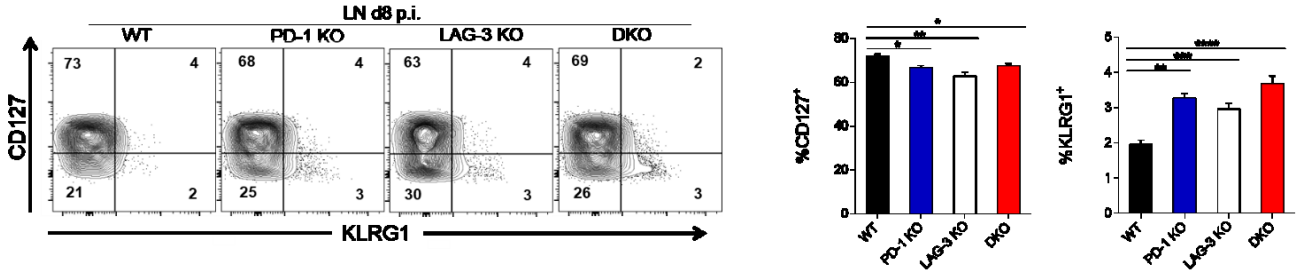

LN d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
CD127
KLRG1
73
4
21
2
68
4
25
3
63
4
30
3
69
2
26
3
%CD127+
%KLRG1+
*
**
***
****

Figure 4.1 PD-1/LAG-3 absence influences $CD8^+$ T cell activation in the acute phase of infection. (A-D) Percent of CD127 and KLRG expression by transferred OT-I cells at day 8 p.i. N = 5 mice / group. Experiments were repeated twice. *$p<0.05$. **$p<0.001$, Kruskal-Wallis test with Dunn's correction for multiple comparisons.

Previous studies have shown that Eomes helps memory cells with long-term persistence, competitive fitness and secondary expansion after re-challenge, but is not a promoter of memory-precursor fate[107]. Thus, we tested the hypothesis that DKO $CD8^+$ T cells' inability to expand would be due to aberrant expression of Eomes during the acute response. To test this, we assessed the expression of the transcription factors T-bet and Eomes in the spleen. Our data show that wild type and single knock out $CD8^+$ T cells had less expression of Eomes when compared to the DKO (Figs 4.2A). Additionally, the DKO cells show a slightly lower expression of T-bet when compared to the other groups.

A.

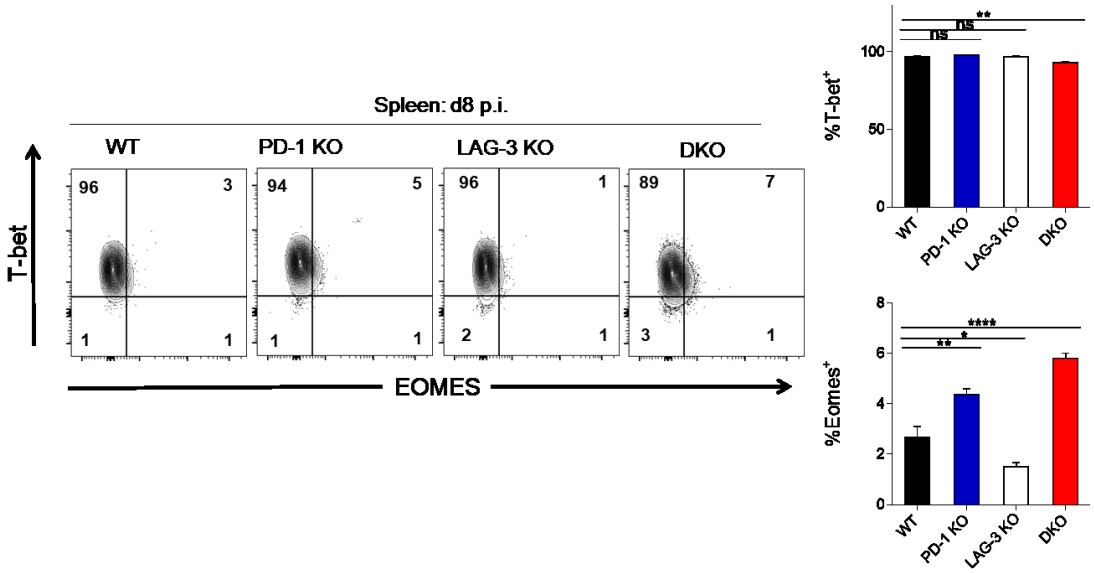

Spleen: d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
T-bet
EOMES
96
3
1
1
94
5
1
1
96
1
2
1
89
7
3
1
%T-bet+
ns
ns
**
100
50
0
%Eomes+
****
*
**
8
6
4
2
0

Figure 4.2 PD-1/LAG-3 absence influences CD8+$^{+}$ T cell activation in the acute phase of infection. (A) Percent of Eomes and T-bet by transferred OT-I cells at day 8 p.i. N = 5 mice / group. Experiments were repeated twice. *p<0.05. **p<0.001, Kruskal-Wallis test with Dunn's correction for multiple comparisons.

Section 4.3: Discussion

Naïve $CD8^+$ T cells have high expression of CD127. Expression of CD127 is lost rapidly after encounter with pathogen. $CD8^+$ T cells responding to antigen downregulate CD127. During a VV-OVA infection CD127 is significantly down-regulated on day 5 and returns to naïve levels by day 7. In our studies we see this behavior in all our groups, however the expression of CD127 is higher in the wild type cells when compared with the other groups.

During the primary response the absence of both checkpoints did not impact activation as shown by the expression of KLRG-1. DKO cells expressed a greater percent of KLRG-1 when compared to the wild type. However, DKO cells retained expression of both markers CD127 and KLRG-1 in almost an equal percent. Previous studies have reported that without the expression of TCF1, $CD8^+$ T cells differentiate into effector cells identified by the expression of KLRG-1. And consistent with those studies we identified $KLRG\text{-}1^{hi}/CD127^{lo}$ expression is increased in DKO T cells.

In terms of clonal expansion, the DKO cells had a greater percent as well as absolute numbers when compared to the wild type. This is consistent for all the organs that we examined.

Chapter V: Cytokines

Section 5.1: Introduction

A plethora of tightly regulated events activates antigen-specific $CD8^+$ T cells responding to infection. This results in their rapid expansion and differentiation into effector cells whose ultimate goal is to help clear infection.[94] During an infection, T cell proliferation and differentiation is modulated a co-stimulatory signals that among other things, help to stimulate the production of cytokines.[179] The main pro-inflammatory cytokines produced during a viral infection are type I IFNs, IFNγ, IL-2, IL-12, IL27 and IL 33 that contribute to proliferation, differentiation and survival of activated $CD8^+$ T cells.[179]

Many factors influence a robust immune response during infection. Although we focus on the importance of PD-1 and LAG-3 for the priming phase of $CD8^+$ T cell, we cannot isolate or undermine the importance of other factors of cytokine production. Past studies have reported that IL-2 signaling during the primary response to infection, primes the formation of competent $CD8^+$ memory T cells that can generate robust secondary responses.[217]

In our studies we used Vaccinia-ova; virus-specific $CD8^+$ T cells control pathogen invasion through the direct killing or virus-infected cells.[218] Past studies have demonstrated that $CD8^+$ T cells responding to viruses have heterogenous effector functions and produce IFN-γ and TNF-α.[39,200,219]

Several studies have proven that in the absence of PD-1 signaling $CD8^+$ T cell function is improved, including cytotoxicity, proliferation and cytokine production.[159,220] Similar results have been reported for LAG-3.[89,126,167,169] But of more relevance to our study, the absence of PD-1 and LAG-3 have a synergic effect in $CD8^+$ T cell effector function.[129,165,221]

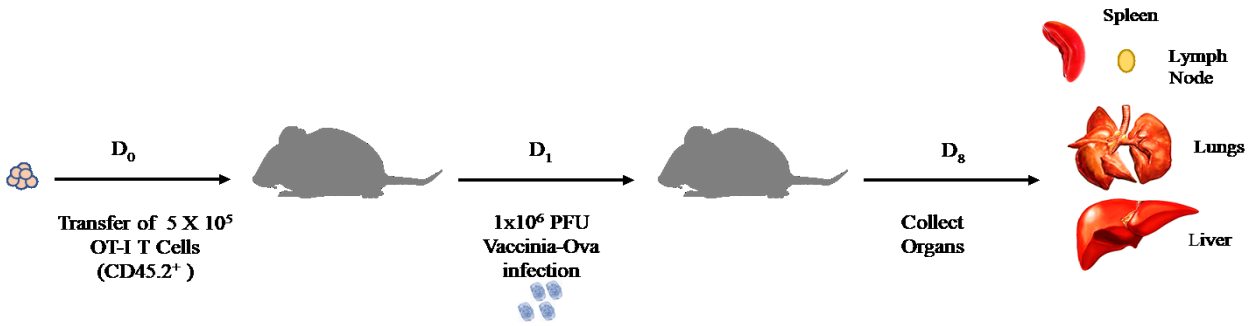
Here we show that a tumor free mouse $CD8^+$ T cell response shows an expected immune response to VV-OVA infection. The response is also effective as shown by the clearance of pathogen.

Section 5.2: Results

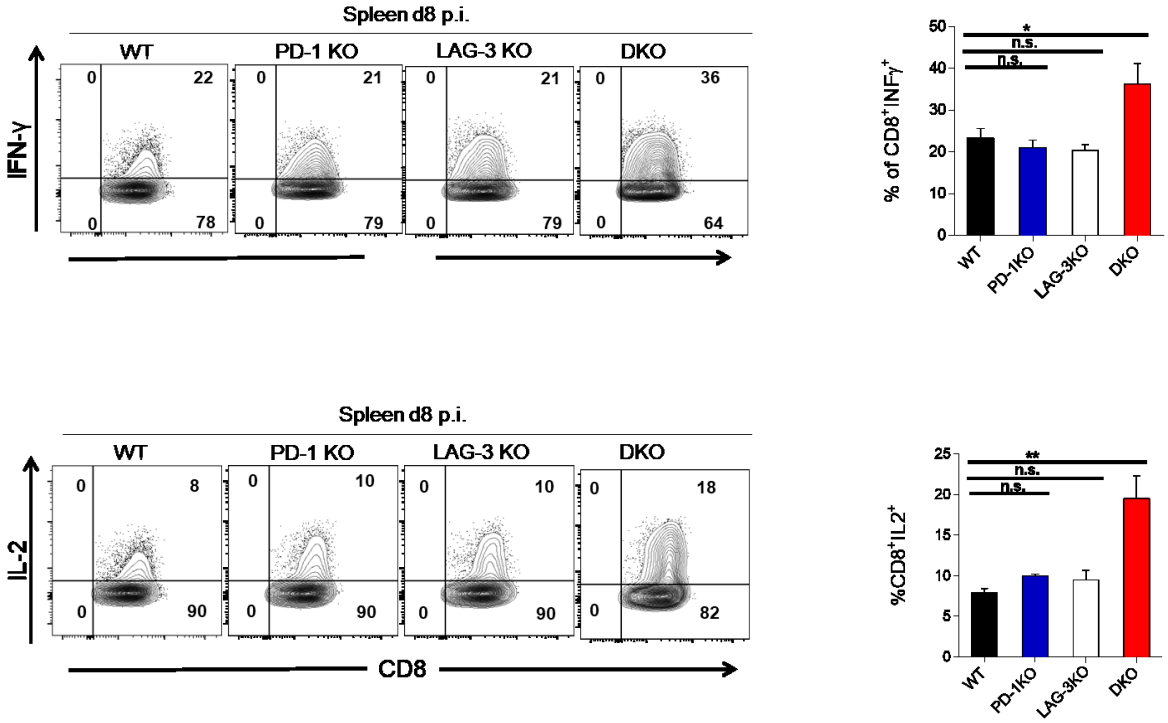
DKO CD8$^+$ T cells have increased polyfunctionality during the acute phase of infection.

Previous experiments using murine tumor models have shown that in the absence of PD-1 and LAG-3, CD8$^+$ T cells increase in number and effector function.[129,165] We next sought to test the hypothesis that in a murine tumor-free model the absence of PD-1 and LAG-3 influences cell effector function. At day 8 p.i. with VV-OVA, DKO OT-I cells showed increased production of cytokines (IFNγ, IL-2) compared to WT or single KO CD8$^+$ T cells (Fig. 5.1A-F). The ability to clear pathogen was also enhanced in the double KO cells (Fig 5.1G). The absence of PD-1 and LAG-3 thus seems to increase terminal differentiation to polyfunctional effector cells during acute infection.

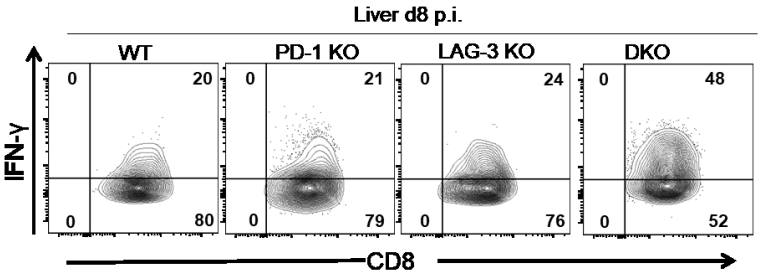
A.

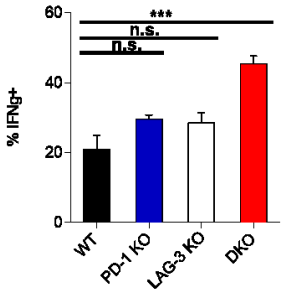

Spleen
Lymph Node
Lungs
Liver
D_0
Transfer of 5 X 10^5 OT-I T Cells ($CD45.2^+$)
D_1
$1x10^6$ PFU Vaccinia-Ova infection
D_8
Collect Organs

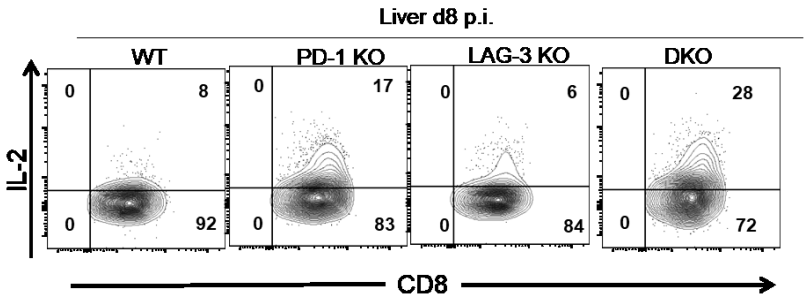
B.

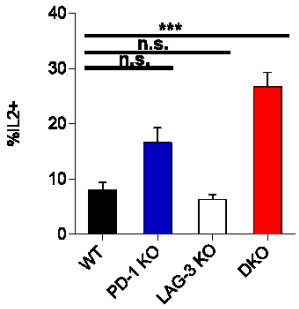

Spleen d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
IFN-γ
0
22
78
21
79
36
64
% of CD8+IFN-γ+
*
n.s.
n.s.
WT
PD-1KO
LAG-3KO
DKO
Spleen d8 p.i.
IL-2
CD8
8
90
10
18
82
%CD8+IL2+
**

C.

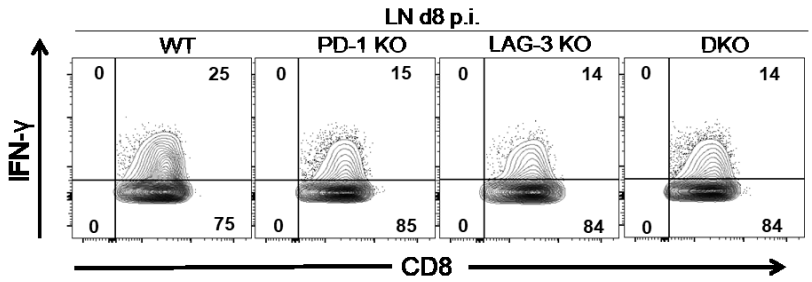

Liver d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
IFN-γ
CD8
0
20
80
21
79
24
76
48
52

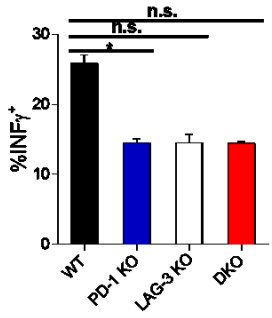

***
n.s.
n.s.
% IFNg+
60
40
20
0
WT
PD-1 KO
LAG-3 KO
DKO

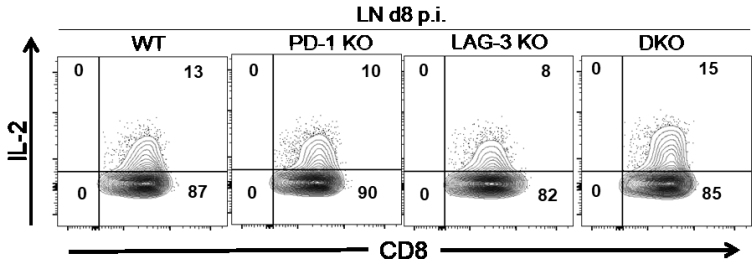

Liver d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
IL-2
CD8
0
8
92
17
83
6
84
28
72

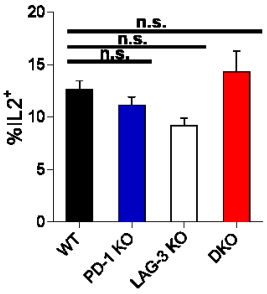

***
n.s.
n.s.
%IL2+
40
30
20
10
0
WT
PD-1 KO
LAG-3 KO
DKO

D.

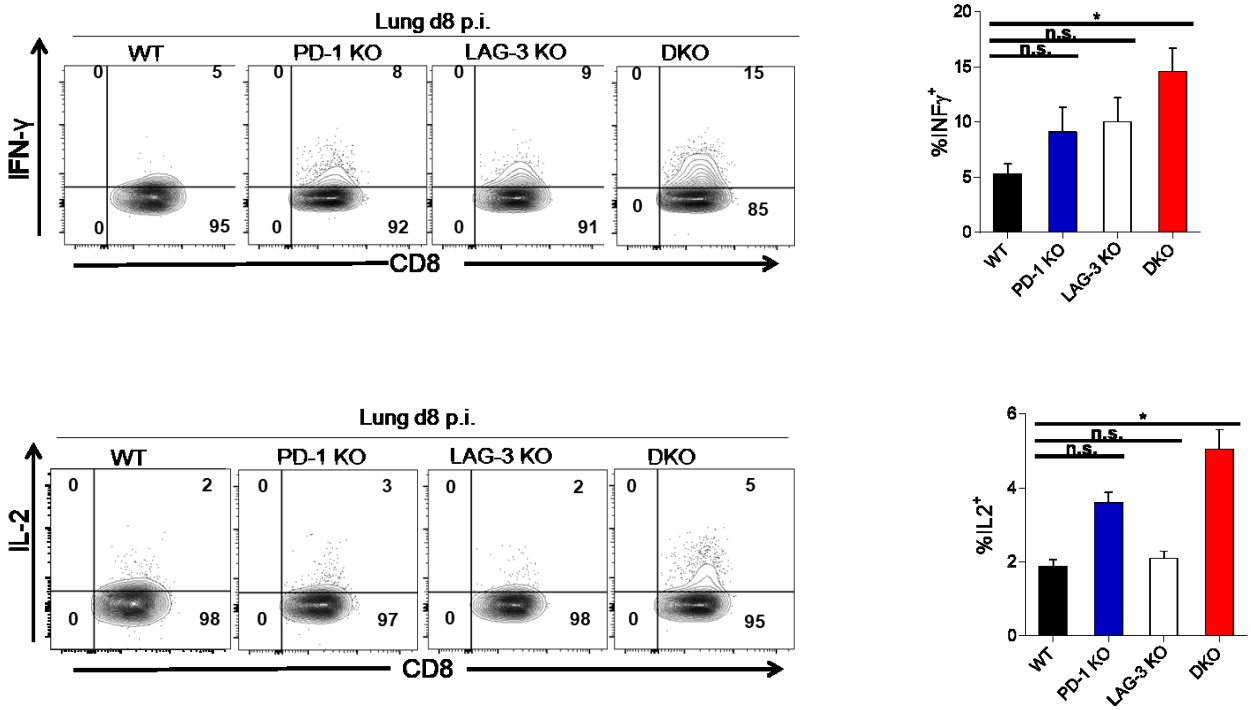

LN d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
IFN-γ
CD8
0
25
75
15
85
14
84
14
84


n.s.
n.s.
*
%INFγ+
30
20
10
0
WT
PD-1 KO
LAG-3 KO
DKO

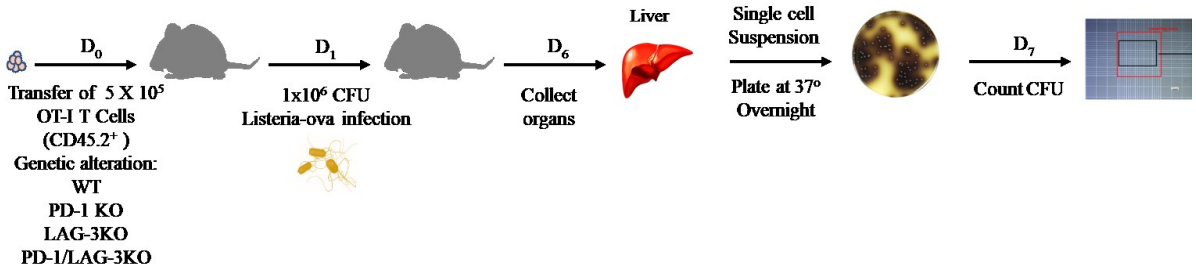

LN d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
IL-2
CD8
0
13
87
10
90
8
82
15
85


n.s.
n.s.
n.s.
%IL2+
20
15
10
5
0
WT
PD-1 KO
LAG-3 KO
DKO

E.


Lung d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
IFN-γ
CD8
0
5
95
8
92
9
91
15
85
%INFγ+
n.s.
n.s.
*
20
15
10
5
0
WT
PD-1 KO
LAG-3 KO
DKO
Lung d8 p.i.
IL-2
2
98
3
97
2
98
5
95
%IL2+
6
4
2

F.


D0
Transfer of 5 X 10^5
OT-I T Cells
(CD45.2+)
Genetic alteration:
WT
PD-1 KO
LAG-3KO
PD-1/LAG-3KO
D1
1x10^6 CFU
Listeria-ova infection
D6
Collect
organs
Liver
Single cell
Suspension
Plate at 37°
Overnight
D7
Count CFU

G.

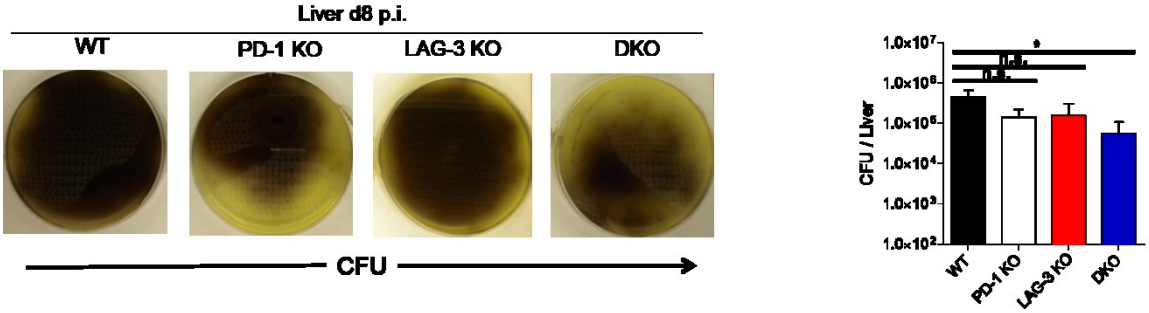

Liver d8 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
CFU
CFU / Liver
1.0x10^7
1.0x10^6
1.0x10^5
1.0x10^4
1.0x10^3
1.0x10^2
*
n.s.
n.s.
WT
PD-1 KO
LAG-3 KO
DKO

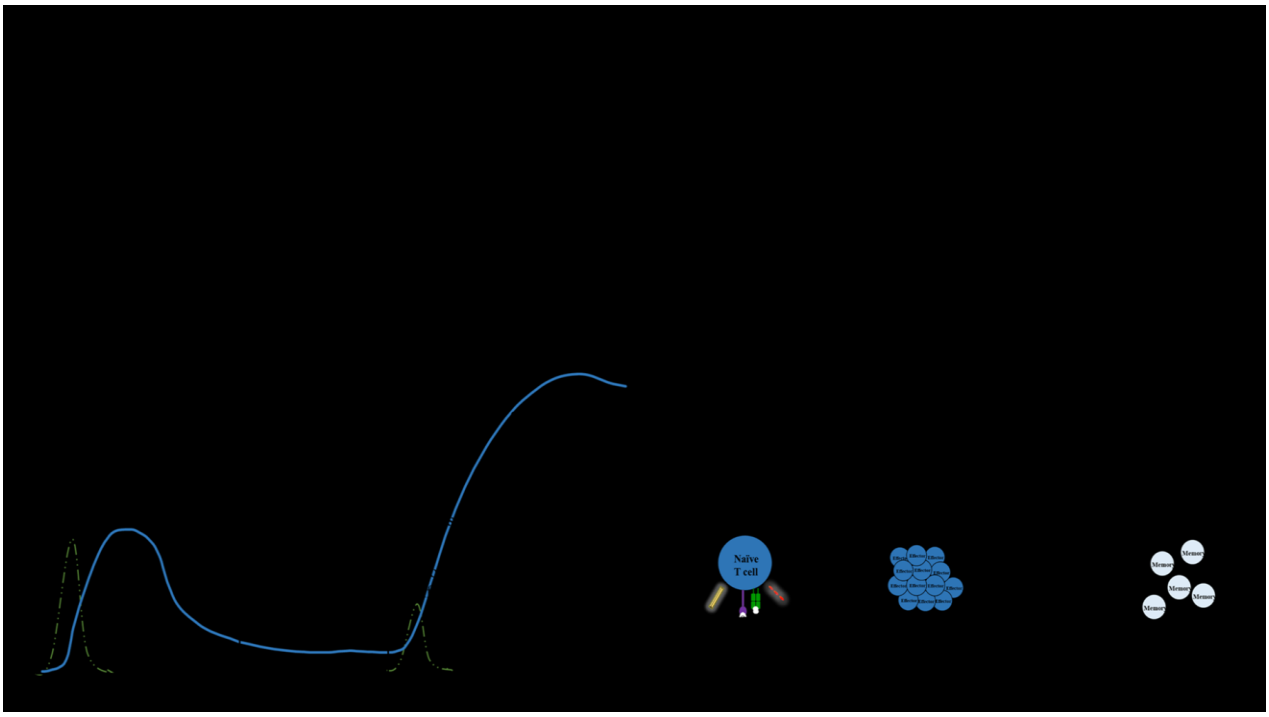
Figure 5.1 DKO OT-I cells have augmented effector function in the acute phase. (A) Schematic representation of organ collection. (B-E) Cytokine expression by WT, PD-1 KO, LAG-3 KO and DKO $CD8^+$ T cells isolated from spleens on day 8 p.i. (F) Schematic representation of pathogen burden assessment. (G) Pathogen clearance in the liver determined by CFU number. N = 5 mice / group. Quantification of LM-OVA CFU in the livers of recipient mice at day 8 p.i. following transfer of OT-1 cells. Experiments were repeated twice. Error bars denote +/- SEM. *$p<0.05$. **$p<0.001$, Kruskal-Wallis test with Dunn's correction for multiple comparisons.

Section 5.3: Discussion

The $CD8^+$ T cell response to pathogens is crucial to clear infection. A major function of $CD8^+$ T cells responding to infection is the lysis of virus-infected cells. A robust and effective response to infection is measured by the production of cytokines including TNF-α, IFN-γ and IL-2.

Consistent with previous studies,[129] our results show that effector function, by the increased expression of cytokines, is enhanced in DKO T cells compared to WT T cells, but extend this finding to a murine model of acute infection. $CD8^+$ T cells seem to behave as expected under a viral infection, not only migrating to different organs but also effectively producing cytokines. We observe that the production of cytokines is homogenous throughout all organs.

We can assess that in the absence of PD-1 and LAG-3, $CD8^+$ T cells have enhanced efficiency clearing a pathogen. We can conclude that during the primary acute response to infection in the absence of PD-1 and LAG-3, antigen-specific $CD8^+$ T cells have increased expansion, activation and effector function.


Naïve
T cell
Memory

Chapter VI: Memory

Section 6.1: Introduction

The concept of immunological memory has been one of the most controversial and disagreed upon.[15,16,56] However there is a consensus in the importance of the immune memory[222] and in the importance of understanding how it is generated and maintained so as to manipulate it for therapeutic benefits.[74,223,224] We know that immunological memory confers to the immune system the ability to remember a response to a specific antigen and upon reencounter, it reacts faster and greater to the same antigen.[93] A key player of the immune system is the $CD8^+$ T cell, especially for combating intracellular pathogens and cancer.[180,225] As mentioned in chapter one, a primary goal of $CD8^+$ T cells in response to infection is the generation of a large number of clones and a second one is the generation of long lasting immunological memory. [179] On the other hand, $CD8^+$ T cell memory has itself two major phenotypes: rapid increase of clone numbers (faster than the first response) and rapid elaboration of effector functions (cytotoxicity and cytokine production).[30]

But before $CD8^+$ T cells have the capacity to behave as memory cells, they must pass through a synchronized and chronological series of steps.[226] A naïve $CD8^+$ T cell circulating in the body can be activated once it encounters and recognizes an antigen. This encounter needs to take place in the context of MHC I antigen presentation, co-stimulation through immune checkpoints and stimulation through receptors for

cytokines.[70,227] The $CD8^+$ T cell memory repertoire is diverse, heterogenous and phenotypic and functional different. Therefore to use a single property to identify or conceptualize memory $CD8^+$ T cells is not only unfair but would give a narrowing perspective.[228] Thus, it results challenging to adhere to a single phenotype or marker as exclusive criteria for a $CD8^+$ T cell that has become a memory cell. [179]

In the same way it would be too narrowing to use a single phenotypic distinction on how precursors of $CD8^+$ T cell memory are formed.[194,229–231] However, some studies have reported the importance of the early stages of $CD8^+$ T cell activation for determining the fate of cells that respond to infection[90] Other studies along the same lines point to the fact that $CD8^+$ T cell memory programming occurs in a short period of time and in a very early stage of activation.[35,202,232]

The success of cancer immunotherapy is measured though several parameters; one of those is the generation of protective immunity. A key way to prevent relapse metastases and recurrence is the formation of immune memory against the primary tumor. [233]

As with VV-OVA, infection with Listeria monocytogenes, elicits an expected $CD8^+$ T cell kinetic response: expansion, contraction and memory development.[208,234] We used an attenuated version that results in an expansion peak at its maximum at day 7.[235,236] Infection with Listeria monocytogenes follows a common pattern as any acute infection and after reaching a peak of expansion, it begins to contract and 5 to 10% of the $CD8^+$ T

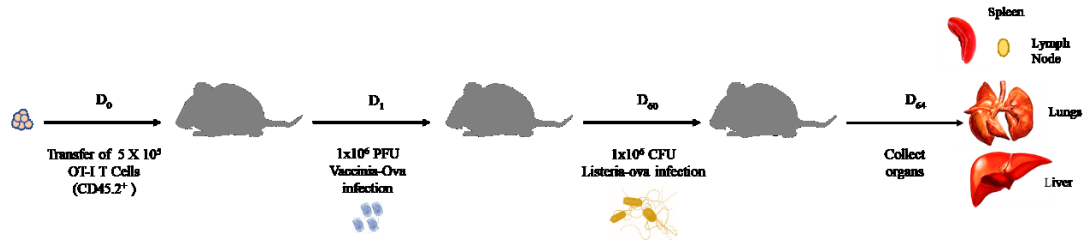
cell clones at the peak transition to memory.[237] However during the recall responses the most robust response is in the liver.[238]

Section 6.2: Results

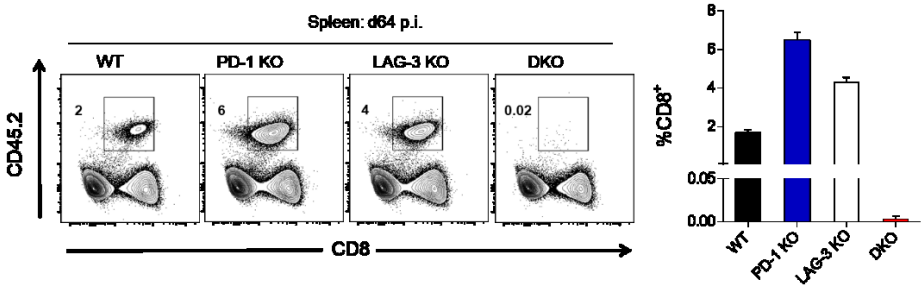
Absence of PD-1 and LAG-3 drives terminal differentiation at the expense of self-renewal.

After assessing the behavior of the $CD8^+$ T cells in the acute phase of infection, we wondered about the quality of memory cells they could produce. We hypothesized that loss of PD-1 and LAG-3 would also generate an enhanced response to secondary challenge in the setting of a non-chronic infection (Fig. 6.3A). Unexpectedly, when we examined the response of DKO T cells to re-challenge with cognate antigen, we found that DKO T cells failed to expand (Fig. 6.1B). To confirm the presence of $CD8^+$ T cells after the expansion plateau, we repeated our experimental setup, let the infection clear and assessed the presence of $CD8^+$ T cells on day 21 after primary infection (Fig.6.1C). At day 21 after the first infection, the frequency of $CD8^+$ T cells was not statistically different when comparing all groups. The observed difference in clonal expansion during the first days of the infection was not sustained.

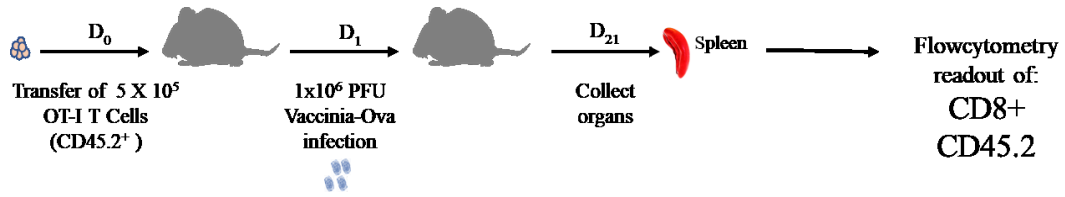
A.


D0
Transfer of 5 X 10^5
OT-I T Cells
(CD45.2+)
D1
1x10^6 PFU
Vaccinia-Ova
infection
D60
1x10^6 CFU
Listeria-ova infection
D64
Collect
organs
Spleen
Lymph
Node
Lungs
Liver

B.


Spleen: d64 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
CD45.2
CD8
2
6
4
0.02
%CD8+
8
6
4
2
0.05
0.00
WT
PD-1 KO
LAG-3 KO
DKO

C.


D0
D1
D21
Spleen
Transfer of 5 X 10^5 OT-I T Cells (CD45.2+)
1x10^6 PFU Vaccinia-Ova infection
Collect organs
Flowcytometry readout of:
CD8+
CD45.2

D.

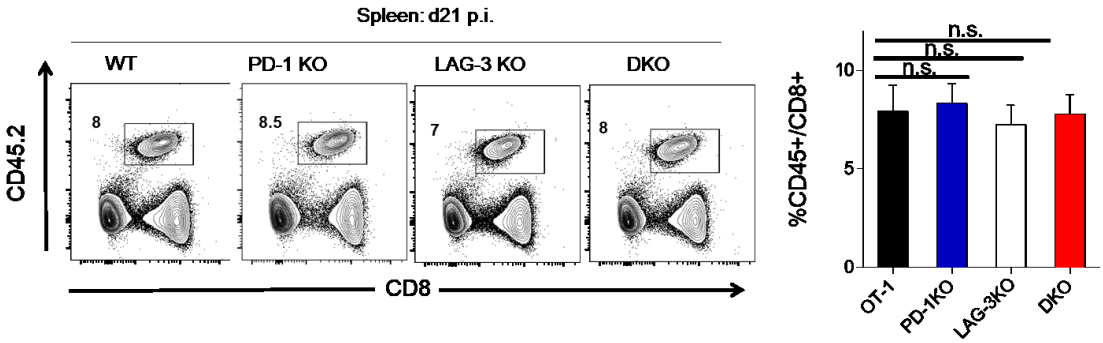
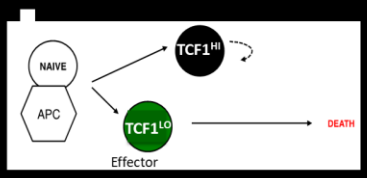
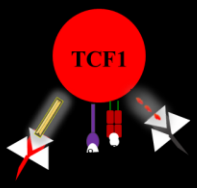

Spleen: d21 p.i.
WT
PD-1 KO
LAG-3 KO
DKO
CD45.2
CD8
8
8.5
7
8
n.s.
n.s.
n.s.
%CD45+/CD8+
10
5
0
OT-1
PD-1KO
LAG-3KO
DKO

Figure 6.1 DKO OT-I cells fail to expand in response to secondary infection. (A) Summary of *in vivo* secondary infection model. OT-I $CD8^+$ T cells of the indicated genotypes are adoptively transferred to congenic mice on day 0, followed by infection with VV-OVA on day 1. Sixty days later, the mice are re-infected with Listeria-ova and the organs are collected at day 64. (B) Representative FACS plots of WT, PD-1 KO, LAG-3 KO and DKO T cells isolated from spleens at day 4 post-re-challenge. Percentages of clonotypic T cells obtained from spleens. (C) Schematic representation of assessment of presence of cells at day 21. (D) Representative plots of $CD8^+$ T cells isolated from the spleen on day 21 and their respective percentages of clonotypic expansion. N = 5 mice / group. Experiments were repeated twice. * $p<0.05$. **$p<0.001$. Kruskal-Wallis test with Dunn's correction for multiple comparisons.

Background:



Expression:



Blockade of PD-1 and LAG-3 increases TCF1 silencing.

Silencing of TCF1 drives terminal $CD8^+$ T cell differentiation and loss of self-renewal, a requisite for memory formation.[44,106,239] (Fig 6.2) To investigate the mechanism underlying loss of secondary recall responses in the absence of PD-1 and LAG-3, we asked whether TCF1 expression was affected during the acute response. Mice were given either no treatment or combined αPD-1/αLAG-3 blockade a day before adoptively transferring cells and infecting them with VV-OVA (Fig. 6.3A). At day 4 p.i., when $TCF1^{hi}$ progenitors self-renew and give rise to differentiated $TCF1^{lo}$ effector cells[43], we found that combined PD-1/ LAG-3 blockade resulted in over-differentiation of $TCF1^{lo}$ cells, with correspondingly increased expression of the activation marker CD44 and effector cell transcription factor IRF4 (Fig 6.3B). We then performed *in vitro* differentiation experiments with antibody-mediated PD-1/LAG-3 blockade in OT-I and P14 T cells (Fig 6.3C). T cells receiving αPD-1 and αLAG-3 treatment had decreased maintenance of $TCF1^{hi}$ progenitors (Fig. 6.3D).

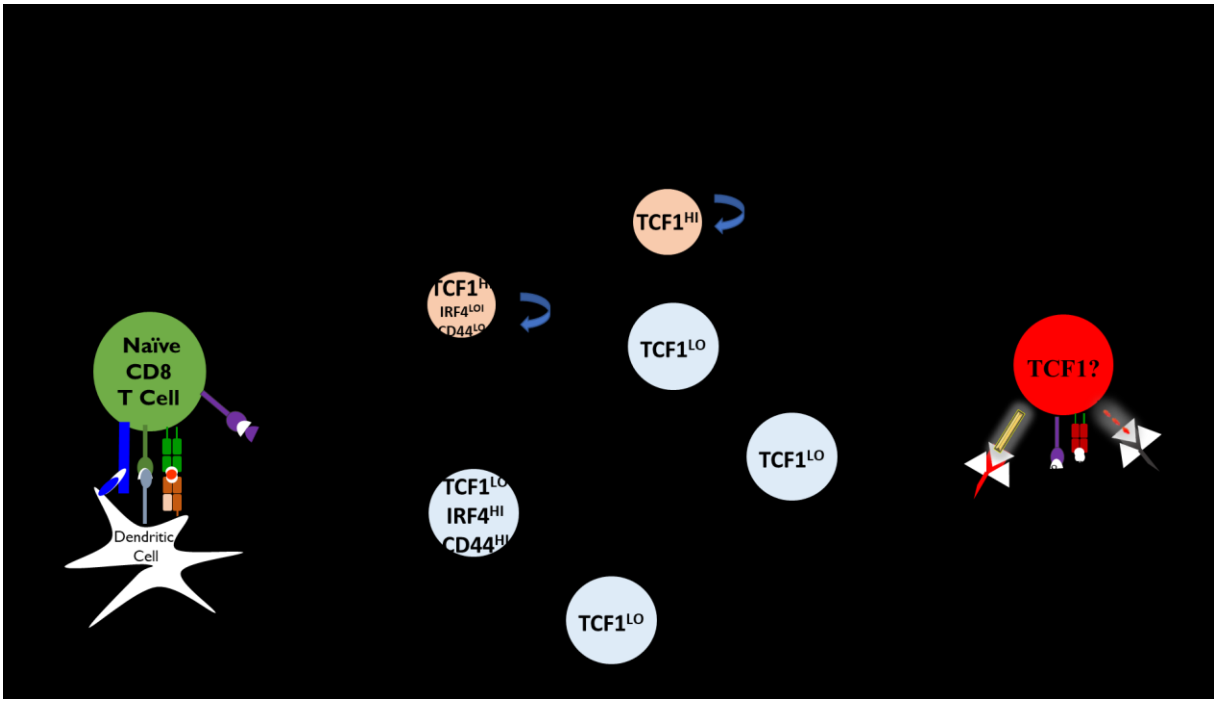
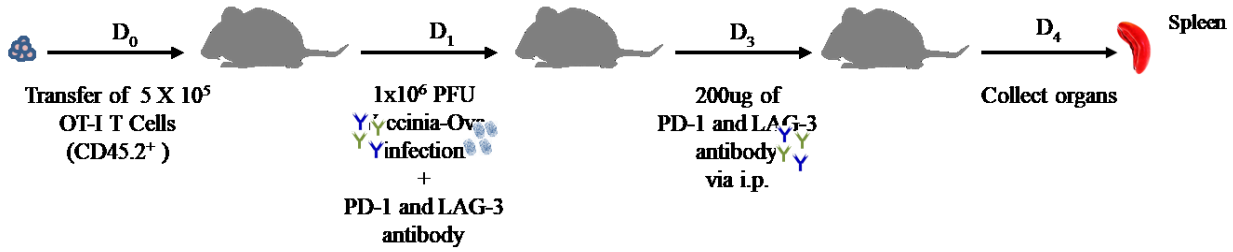
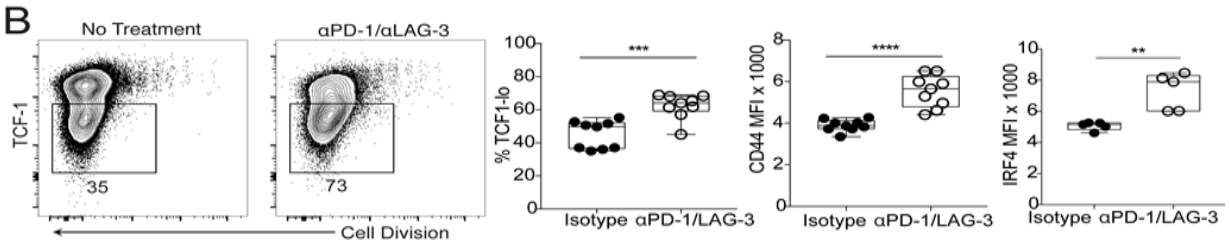

Naïve
CD8
T Cell
Dendritic
Cell
TCF1HI
TCF1LO
IRF4HI
CD44HI
TCF1LO
TCF1LO
TCF1LO
TCF1?

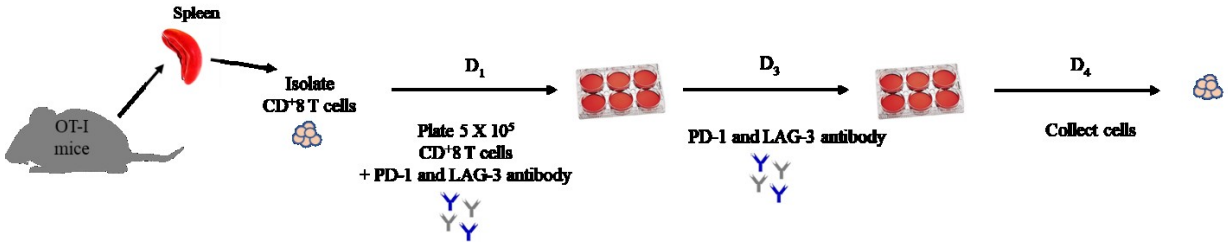
Fig 6.2 Schematic representation of TCF1 silencing and memory formation. TCF1 is a transcription factor that is key in regulating T cell memory. TCF1 marks and maintains $CD8^{+}$ T cells with capacity for self-renewal in acute and chronic infections.$TCF1^{lo}$ cells only give rise to more TCF^{lo} cells acutely, and lack self-renewal properties after pathogen clearance. In the other hand $TCF1^{hi}$ have the capacity of producing $TCF1^{lo}$ cells while self-renewing the $TCF1^{hi}$ pool. Once pathogen is cleared, the $TCF1^{hi}$ cells have self-renewal properties and remind quiescent. Thus, entering the central memory T cell pool. In other words, as reported by the Reiner lab, silencing expression of TCF1 marks loss of self-renewal, and such silencing requires cell division.

A.


D0
D1
D3
D4
Spleen
Transfer of 5 X 10^5 OT-I T Cells (CD45.2+)
1x10^6 PFU
Vaccinia-Ova
infection
+
PD-1 and LAG-3 antibody
200ug of PD-1 and LAG-3 antibody via i.p.
Collect organs


B
No Treatment
αPD-1/αLAG-3
TCF-1
35
73
Cell Division
% TCF1-lo
CD44 MFI x 1000
IRF4 MFI x 1000
***
****
**
Isotype
αPD-1/LAG-3

C.


Spleen
OT-I mice
Isolate CD+8 T cells
D1
Plate 5 X 10^5 CD+8 T cells + PD-1 and LAG-3 antibody
D3
PD-1 and LAG-3 antibody
D4
Collect cells

D.

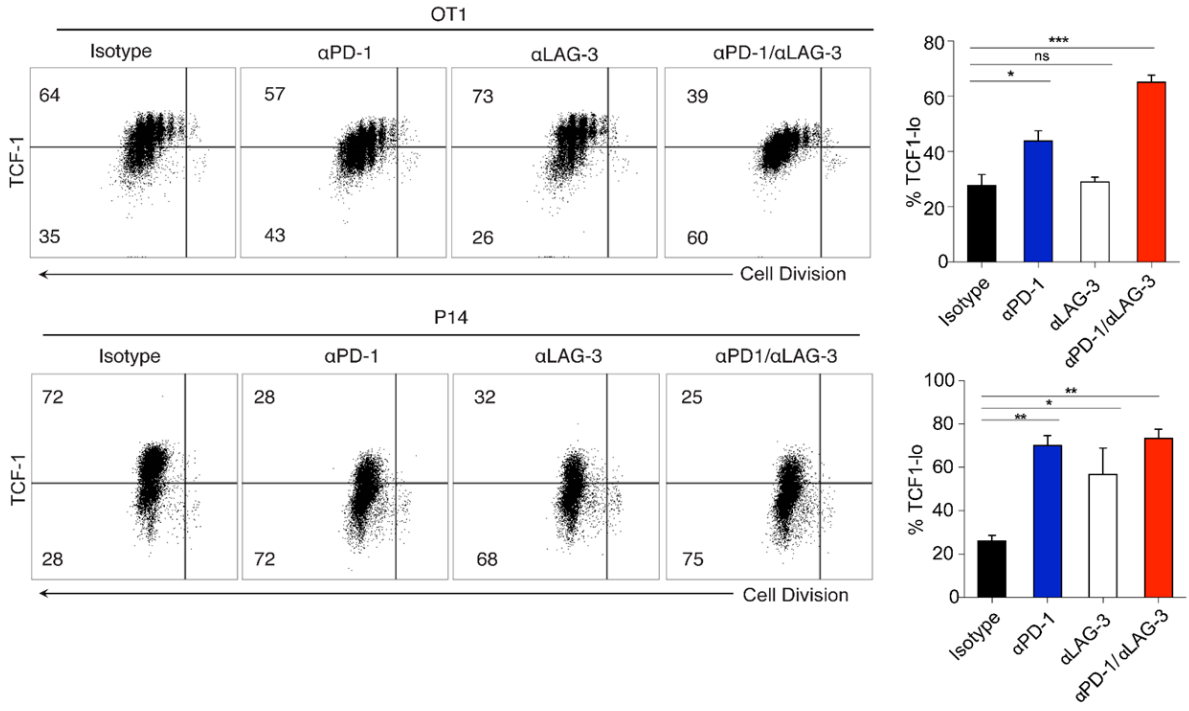
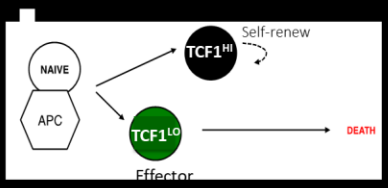

OT1
Isotype
αPD-1
αLAG-3
αPD-1/αLAG-3
64
57
73
39
35
43
26
60
TCF-1
Cell Division
P14
Isotype
αPD-1
αLAG-3
αPD1/αLAG-3
72
28
32
25
28
72
68
75
TCF-1
Cell Division
% TCF1-lo
ns
Isotype
αPD-1
αLAG-3
αPD-1/αLAG-3
% TCF1-lo
Isotype
αPD-1
αLAG-3
αPD-1/αLAG-3

Figure 6.3. Blockade of PD-1 and LAG-3 increases TCF1 silencing. (A) Schematic representation of *in vivo* checkpoint blockade. OT-I $CD8^+$ T cells were adoptively transferred to congenic recipient mice on day 0, followed by infection on day 1 and PD-1/LAG-3 antibody blockade on day 1 and 3. Spleens were collected on day 4. (B) Left: TCF1 expression versus cell division for transferred OT-I cells. Middle: Quantification of percent $TCF1^{lo}$ cells. Right: CD44 and IRF4 expression on transferred OT-I cells N = 5 mice / group. Experiments were repeated twice. (C) Schematic representation of *in vitro* checkpoint blockade. OT-I $CD8^+$ T cells positively selected and plated with Ova peptide and PD-1/LAG-3 antibody blockade on day 1 and 3. Cells were collected on day 4. (D) FACS plots and quantification of TCF1 lo of $CD8^+$ treated with either Isotype, αPD-1, αLAG-3, or αPD-1/αLAG-3 antibody at day 4 of culture. OT-I (upper) or P14 (lower). * $p<0.05$. ** $p<0.01$. *** $p<0.001$. n.s. not significant. Kruskal-Wallis test with Dunn's correction for multiple comparisons.

Background:

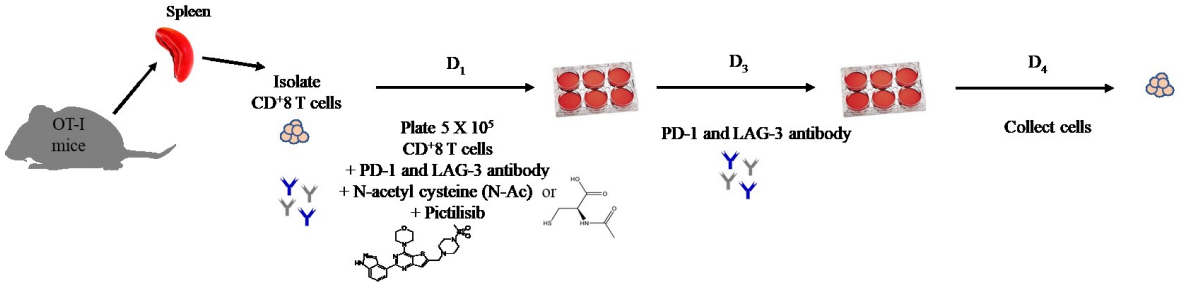


Memory CD8
T cell:

Altering the metabolism of CD8+ T cells in the absence of PD-1 and LAG-3 changes the phenotype of TCF1.

PD-1 blockade acts through CD28 signaling to enhance anti-tumor immunity,[124,240] and CD28 induces PI3K activity as well as downstream anabolic pathways.[241] A constellation of PI3K-driven and anabolic processes mediates repression of TCF1 during T cell differentiation.[175] We hypothesized that scavenging anabolism-associated reactive oxygen species (ROS) or blocking class I PI3K signaling might blunt the over-differentiation phenotype induced by combined PD-1/LAG-3 blockade (Fig. 6.4A). Indeed, perturbing these anabolic pathways in the context of combined PD-1/LAG-3 blockade decreased the frequency of cells undergoing repression of TCF1 (Fig. 6.4B).

A.


Spleen
OT-I mice
Isolate CD+8 T cells
D1
Plate 5 X 10^5 CD+8 T cells
+ PD-1 and LAG-3 antibody
+ N-acetyl cysteine (N-Ac) or
+ Pictilisib
D3
PD-1 and LAG-3 antibody
D4
Collect cells

B.

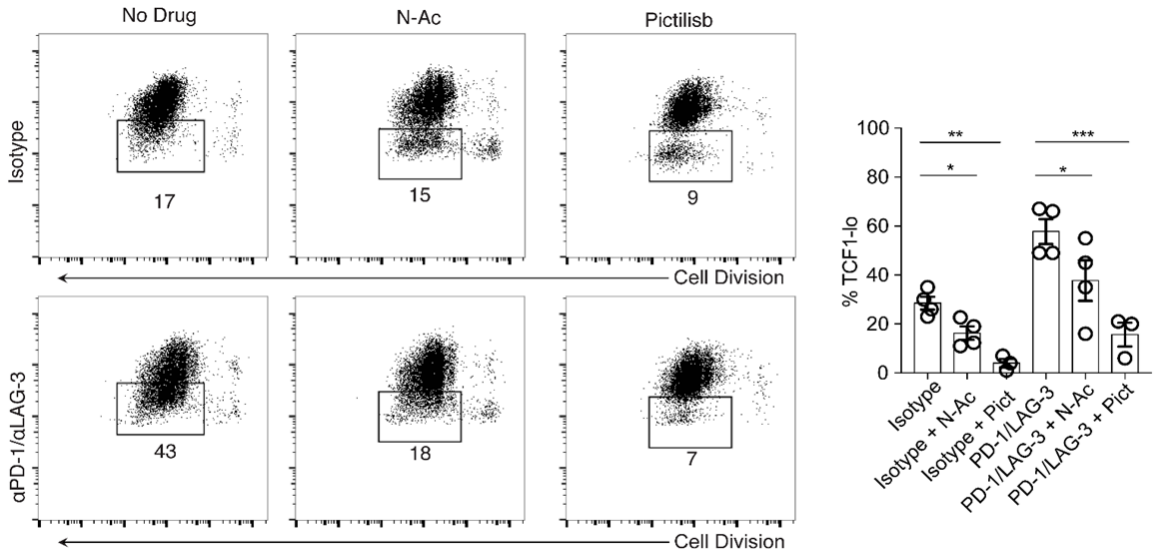

No Drug
N-Ac
Pictilisb
Isotype
17
15
9
Cell Division
αPD-1/αLAG-3
43
18
7
Cell Division
% TCF1-Io
100
80
60
40
20
0
**
*
***
*
Isotype
Isotype + N-Ac
Isotype + Pict
PD-1/LAG-3
PD-1/LAG-3 + N-Ac
PD-1/LAG-3 + Pict

Figure 6.4. Perturbing ROS and PI3k rescues the expression of TCF1 in the absence of PD-1 and LAG-3 in $CD8^+$ T cells. (A) Schematic representation of *in vitro* checkpoint blockade. OT-I $CD8^+$ T cells positively selected and plated with on day 1 with OVA peptide, N-acetyl cysteine (ROS scavenger), or Pictilisib (Class I PI3K inhibitor) and PD-1/LAG-3 antibody blockade on day 1 and 3. Cells were collected on day 4. (B) ROS scavenging or inhibition of Class I PI3K activity rescues over-differentiation induced by checkpoint blockade. Left: P14 cells are treated with either no drug, N-acetyl cysteine (ROS scavenger), or Pictilisib (Class I PI3K inhibitor). Right: quantification of $TCF1^{lo}$ cell frequency at day 4 of culture. * $p<0.05$. ** $p<0.01$. *** $p<0.001$. n.s. not significant. Kruskal-Wallis test with Dunn's correction for multiple comparisons.

Section 6.3: Discussion

As previously reported, TCF1 silencing determines the fate of T cells differentiation.[43] Consistent with those studies, we show that in the absence of PD-1 and LAG-3 there is a higher percentage of $TCF1^{lo}$ cells, which has been associated with terminal differentiation of $CD8^+$ T cells.[52] Additionally, other studies report that differentiating T cells show metabolic changes such as glycolysis and ROS production that are coupled to the silencing of TCF1 or to the manipulation of PD-1.[175,242] We report that in the absence of PD-1 and LAG-3, $CD8^+$ T cells failure to expand during the secondary response to acute infection phase. This might be due to the higher TCF1 silencing we observed during the early phases of T cell differentiation that take place following primary acute infection.

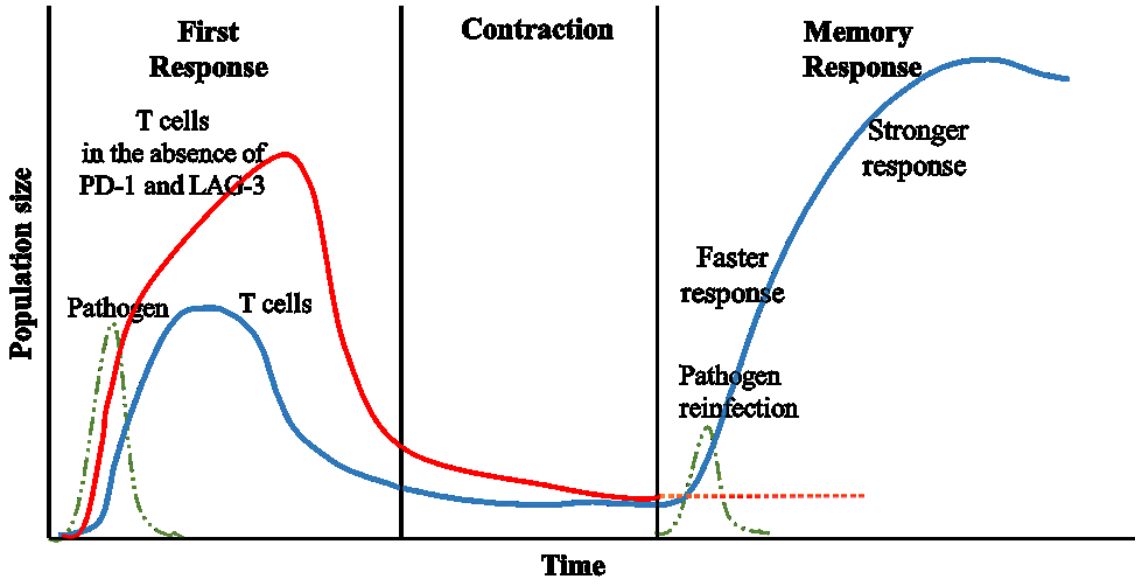
We show that T cells fail to expand in response to re-challenge with acute infection using an OT-1 KO *in vivo* model, supporting our hypothesis that the immune checkpoints PD-1 and LAG-3 influence $CD8^+$ T cell differentiation.[101] We moved our studies *in vitro* to examine the mechanism of how this happens. We report that blocking PD-1 and LAG-3 silences the expression of TCF1, and $CD8^+$ T cells show greater potential to differentiate into effector cells at the expense of self-renewal and memory formation in murine OT-1 and P14 cells. It would be valuable to assess TCF1 expression and its influence in long-term memory in the absence and presence of other immune checkpoints.

Our results suggest that loss of PD-1 and LAG-3 in $CD8^+$ T cells results in greater effector differentiation at the expense of long-term T cell memory formation due to increased silencing of TCF1. In chronic-active states, such as cancer, greater effector differentiation could occur at the expense of self-renewal of the active progenitors that continually give rise to new effector cells. After acute infection, cells activated without immune checkpoints might fail to expand during re-challenge, possibly due to enhanced frequency of TCF1 silencing during the acute phase.

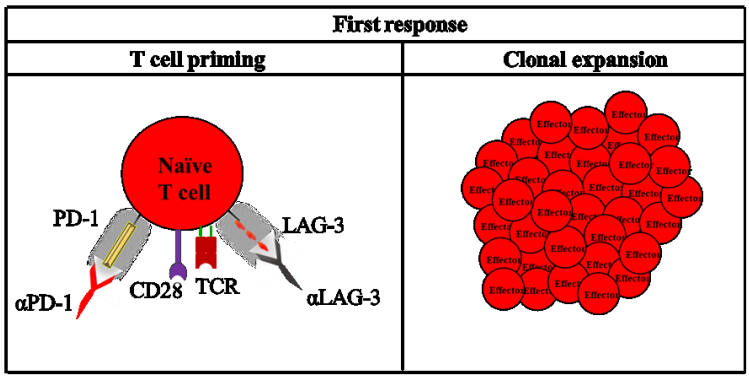
Chapter VII: Discussion

The present data show that the immune checkpoints PD-1 and LAG-3 influence $CD8^+$ T cell differentiation. We show that in the absence of PD-1 and LAG-3, $CD8^+$ T cells lead to increased T cell effector function during the acute response to infection. However, this does not lead to an enhanced secondary response. Rather than being enhanced, the secondary response is nonexistent as the double knockout cells seem unresponsive to re-challenge (Fig 7.1). Although during the primary response T cells responded effectively and were able to clear pathogen infection, they were not effective in generating long term memory. Analysis of T cells showed that in the absence of PD-1 and LAG-3, $CD8^+$ T cells show greater potential to differentiate into effector cells at the expense of self-renewal and memory formation.

A.


First
Response
Contraction
Memory
Response
T cells
in the absence of
PD-1 and LAG-3
Stronger
response
Faster
response
Pathogen
T cells
Pathogen
reinfection
Population size
Time

B.


First response
T cell priming
Clonal expansion
Naïve
T cell
PD-1
LAG-3
αPD-1
CD28
TCR
αLAG-3

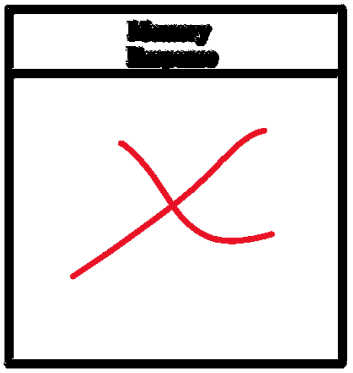


Figure 7.1. Kinetics of memory CD8$^+$ T cell. T cell differentiation during an acute response in the absence of PD-1 and LAG-3. (A) Kinetic curve of T cell expansion during a viral infection in red in absence of PD-1 / LAG. (B) After pathogen encounter CD8++ T cells expand greatly but fail to expand upon re-challenge.

As in previous research our results show enhanced T cell function in double knockout T cells, but in an acute infection mouse model. However, unlike reported results of single PD-1 knockout in a chronic model; in our model double knockout T cells neither produce cytokines nor behave as exhausted-like cells, and they also fail to expand upon re-challenge. [129,211,243] As previously reported, TCF1 silencing determines the fate of T cells.[176] We show that in the absence of PD-1 and LAG-3 there is higher percent of TCF^{lo} T cells, which as reported by Wen-Hsuan[52] leads to terminal differentiation.

Additionally it has been reported that differentiating T cells show metabolic changes that are coupled to the silencing of TCF1 or to the manipulation of PD-1(Fig 7.2).[160] This supports our findings that in the absence of PD-1 and LAG-3 T cells fail to expand due to the higher TCF1 silencing observed during the early phases of T cell differentiation.

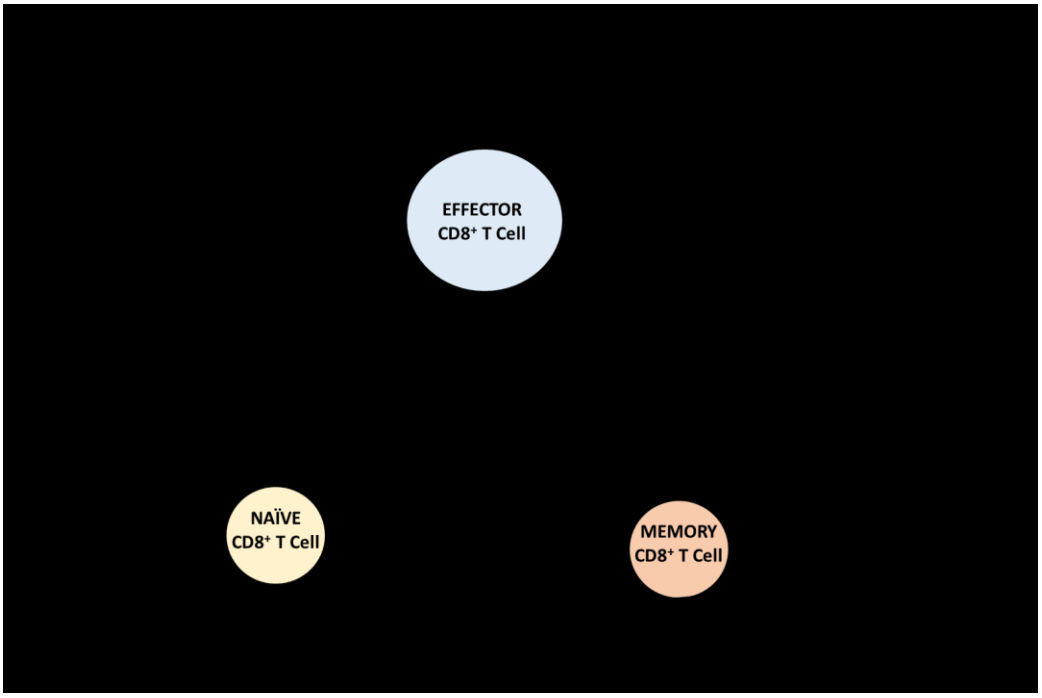

EFFECTOR
CD8+ T Cell
NAÏVE
CD8+ T Cell
MEMORY
CD8+ T Cell

Figure 7.2. Metabolic changes coupled to the silencing of TCF1 in $CD8^+$ T cell differentiation. Once T cells are activated, they increase in size and undergo deep metabolic changes. They transition from a quiescent state to a more active one, metabolically they transition from oxidative phosphorylation (OXPHOS) to glycolysis. Signals from growth factor cytokines like interleukin-2 (IL-2) and the ligation of costimulatory CD28 help activate glycolytic pathways by inducing the phosphoinositol 3-kinase (PI3K)-dependent activation of AKT. Mamalian target of rapamycin (mTOR) is a key regulator of translation and cell size.

Studies show that for clonal expansion, $CD8^+$ T cells do not need a stable interaction with dendritic cells, as a result $CD8^+$ T cells differentiate into effector cells. However, if such interaction does not happen, memory cells are affected. This is a crucial observation for our model, since such findings support that the memory potential of $CD8^+$ T cells can be programmed very early in the response phase, as early as the first 24 hours of priming.[244]

Several recent findings suggest that restraining anabolic signals in parallel with checkpoint blockade can improve immunotherapeutic response for multiple cancers as well as augment tumor control following adoptive transfer therapy.[148,241,245–247] Moreover, construction of chimeric antigen receptor T cells with signaling domains that promote oxidative phosphorylation over aerobic glycolysis promotes more effective tumor eradication (Fig 7.3).[248] Our results support future immunotherapy design that balances combinatorial checkpoint blockade therapies with treatments to restrain anabolic signaling and provide context for the seemingly paradoxical findings that combination immunosuppressive/checkpoint blockade treatment might promote better tumor control.

Metabolic changes coupled to the silencing of TCF1

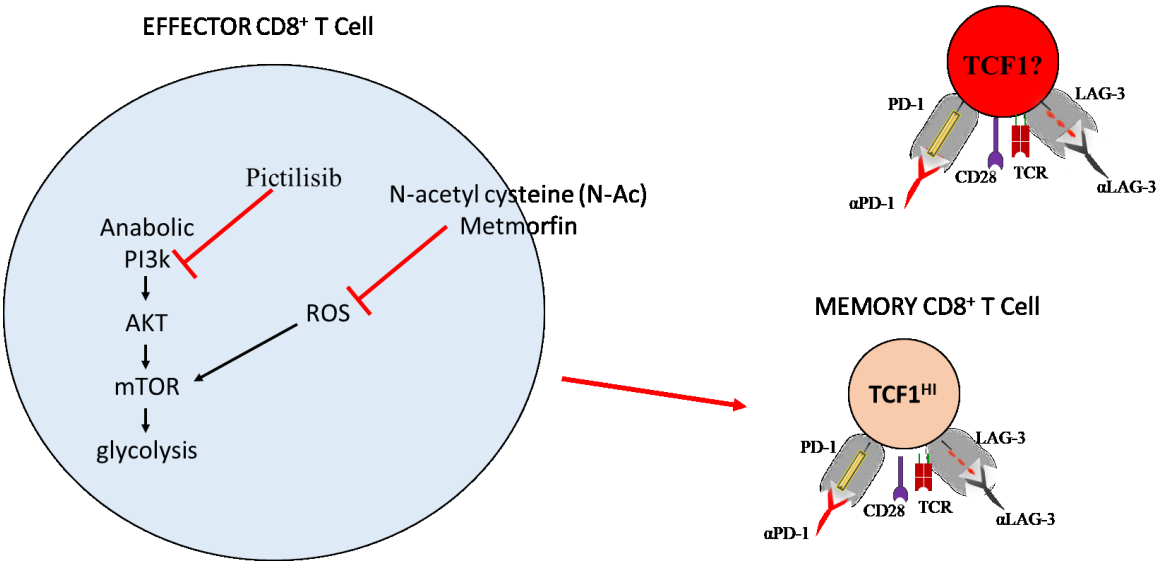


Figure 7.3. Metabolic changes coupled to the silencing of TCF1 in CD8$^+$ T cells can be manipulated. We hypothesized that scavenging anabolism-associated reactive oxygen species (ROS) or blocking class I PI3K signaling might blunt the over-differentiation phenotype induced by combined PD-1/LAG-3 blockade. Therefore, we used pictilisib and N-AC, to interfere with the signaling of PI3k and ROS respectively.

These data are important in understanding the differentiation of $CD8^+$ T cells during acute response in the context of immune checkpoints. Understanding how PD-1 and LAG-3 influence the formation of memory can help design combinatorial cancer therapies that focus on vaccines thus reducing the potential of tumor development in case of relapse. Here, we show how the combined absence of PD-1 and LAG-3 impacts immune responses in an acute infection and re-challenge model. We show that the combined loss of PD-1 and LAG-3 leads to defective memory cell formation and poor recall responses to secondary challenge. We then assess the mechanism underlying this deficit and characterize the role of TCF1 expression in regulating memory cell differentiation. There are several models proposed for the understanding of how $CD8^+$ T cells arise. At first it was reported that some effector $CD8^+$ T cells had the ability of either retaining or re-expressing IL-7R at the peak of the immune response. Those cells then survived the contraction phase and gave rise to memory cell population. We have used all those reports to do our studies to assess the ability of cells to get activated and functional in during the acute response.

Conclusions

- Prior findings in tumor models support that in our results T cell proliferation and effector cell differentiation is enhanced by the double deficiency of PD-1 and LAG-3 during acute infection.
- Our results show that in contrast to the phenotype of enhanced primary expansion, DKO T cells are impaired in secondary expansion.
- The continued capacity for antigen-elicited proliferation and self-renewal alongside production of differentiated cells has been linked to persistent expression of TCF1 by CD8+ T cells.
- Anabolic PI3K signaling causes FoxO1 inactivation and TCF1 silencing, while PD-1 signaling opposes anabolic PI3K/AKT/mTOR, the subsequent defect in expansion of DKO T cells may relate to an inability to maintain expression of TCF1 and the associated capacity for proliferation and self-renewal.
- Whether PD-1/LAG-3 blockade is functioning through TCF1 silencing will require further investigation.
- Emerging evidence suggests that control of acute, persistent, and chronic-active infections relies on stem- and progenitor-like T cells that have the paradoxical capacity to self-renew while producing more differentiated daughter cells
- The role of inhibitory receptors in opposing costimulatory signals and anabolic metabolism suggests that inhibitory pathways may have evolved as an obligatory counterbalance for the dichotomous processes of immunity: anabolism and catabolism; differentiation and self-renewal; function and durability.

- Restraining anabolic signals in parallel with checkpoint blockade could improve immunotherapeutic response for multiple cancers as well as augment tumor control following adoptive transfer therapy
- Our results support future immunotherapy design that balances combinatorial checkpoint blockade therapies with treatments to restrain anabolic signaling and provide context for the seemingly paradoxical findings that combination immunosuppressive/checkpoint blockade treatment might promote better tumor control

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CURRICULUM VITAE
The Johns Hopkins University School of Medicine

Maria Augusta Carrera-Haro March 1st 2019

Educational History

PhD expected	2019	Program in Immunology Mentor: Charles Drake MD., PhD.	Johns Hopkins School of Medicine
B.S.	2012	Biotechnology, Summa cum laude	Stevenson University

Other Professional Experience

- Graduate Research Student. Dr. Drake Laboratory, September 2013 - present.
    - My thesis research focuses in CD8+ T cell memory formation in the context of the immune checkpoints PD-1 and LAG-3. I study the influence PD-1 and LAG-3 CD8+ T cell differentiation during the priming phase of the acute response.
- Intern, Department of Oncology at Dr. David Loeb's Laboratory, May – September 2011 and summer 2012.
    - Assisted in the analysis of a clinical trial studying the combination of temsirolimus and liposomal doxorubicin for the treatment of patients with recurrent or refractory sarcomas.
    - Investigate the status of mTOR signaling in pretreatment biopsy samples and to determine the pharmacokinetics of temsirolimus in trial participants. My work showed that the expression of AKT/pAKT and S6/pS6 were variable, and that mTORC1 signaling is more highly activated in sarcoma than mTORC2.
- Intern, Johns Hopkins University, Department of Oncology at Dr. Jonathan Powell's laboratory, assisting Dr. Christopher Gamper, summer 2009 and 2010.
    - I studied the role of DNMT3a in regulating the methylation of the interferon-gamma (IFN-γ) promoter during T cell differentiation. I helped show that genetically modified mice which do not express DNMT3a had a resulting deficiency in their immune system.
- Mentor for MERIT Health Leadership Academy, summer 2016.
    - Mentored one high school student.
    - Designed a small project for the student to learn scientific concepts and research techniques.
- Biochemistry teaching assistant Stevenson University, spring 2010.
    - Held twice per week classes with reviewing and clarifying concepts learned in class.

    - Designed reviews with working biochemistry problems to understand concepts and prepare students for tests.
- Instructional Anatomy and Physiology, General Chemistry and microbiology tutor for Algebra for Baltimore City Community College, June 2007 – May 2009.
    - Tutored one on one to students.
- Anatomy and Physiology lab assistant at Baltimore City Community College. November 2008 – May 2009.
    - Setup anatomical models and experiments for anatomy lab classes
    - Kept the the laboratory organized, clean and troubleshoot faulty equipment
    - Corrected quizzes and helped prepare the class
- Algebra teaching assistant at Baltimore City Community College, August 2008 - December 2008.
    - Assisted in designing weekly small practice test for students to reinforce concepts from the classroom.
    - Review concepts learnt in class and practiced algebra problems.
    -

Publications:

- K.A. Thornton, A.R. Chen, M.M. Trucco, P. Shah, B.A. Wilky, N. Gul, **M.A. Carrera-Haro**, M. Fogle Ferreira, U. Shafique, J.D. Powell, C.V. Meyer, D.M. Loeb. 2013 A dose-finding study of temsirolimus and liposomal doxorubicin for patients with recurrent and refractory bone and soft tissue sarcoma. Cancer Therapy.

- Sarah McCormick, Nagaraj Gowda, **Maria Carrera**, Jessie Fang, Nicolla Heller. 2015. SOCS1 regulates IL-4-activated IRS-2 tyrosine phosphorylation in monocytes and macrophages via the proteasome. The Journal of Immunology.

Awards:

- 2018 AAI Late-Breaking Poster Award

Posters:

- Carrera-Haro MA, Reiner SL, Drake CG (2018). Immune Checkpoints Acting as Gate-keepers of T Lymphocyte Self-renewal. American Association of Immunology, Austin TX. May 8th 2018.

Leadership activities

- Yale Ciencia Academy fellow January December 2016 – December 2017
    - Year-long program with diverse leadership building activities.
- Science Alliance Leadership Training (SALT) Fellow of the New York Academy of Sciences, July 2017.
    - Learned leadership skills and management of conflict.
- Co-President of the Student Council for the Immunology Graduate Program at the Johns Hopkins University School of Medicine, June 2016 - June 2017.
    - Lead student in other positions in the council.
    - Worked in updating and improving guidelines for elections and expectations of student council members.
- Interim President of the Student Council for the Immunology Graduate Program at the Johns Hopkins University School of Medicine, March 2016 - June 2016.
    - Created and lead a group of student hosts giving them guidelines to how to write the bio sketches of invited faculty and how to advertise the talks. Helped develop a schedule of faculty speakers from outside of Johns Hopkins.
    - Wrote and instituted guidelines for elections and defined the duties and expectations for the other council members.
    - Organized social events like happy hours for students and faculty in the program.
- Assistant of the President of the Student Council for the Immunology Graduate Program at the Johns Hopkins University School of Medicine, May 2015 - March 2016.
    - Assisted president in the implementation of guidelines for leadership roles in the program
- Reviewed research grants for women and minorities in the School of Medicine for the Office of Student Diversity during the 2015 - 2016 academic year.
- Journal club leader for the Immunology Graduate Program at Johns Hopkins University School of Medicine, November 2014 - May 2015
    - Organized student speaker schedule, ordered food, advertised the meeting and helped with the distribution of the scientific paper in a weekly bases.

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